THE UNIVERSITY OF ALBERTA

A STUDY OF THE MORPHOLOGY AND FUNCTION OF MESOSOMES IN *NEUROSPORA CRASSA*

by

JOSEPH R. KOKE

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled A study of the morphology and function of mesosomes in Neurospora crassa, submitted by Joseph R. Koke in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

ABSTRACT

Investigations into the structure and function of mesosomes of Neurospora crassa were made using electron microscopial, histochemical, biochemical, and radiotracer techniques. This study is divided into two parts, morphological investigations and functional investigations.

Morphological investigations were performed by light and electron microscopy on Neurospora at various growth stages. Various fixatives and embedding procedures were employed, and attempts were made to determine whether or not mesosomes of Neurospora are real structures or are artifacts of preparatory techniques. It was found that mesosomes are seen more frequently at some stages than others, that there may be two kinds of mesosomes, and that they are seen most commonly in cells that have been fixed in glutaraldehyde followed by OsO_4. The effect of various fixatives on the appearance of mesosomes suggests that the morphology of these structures may be determined by the type of fixative employed.

Functional investigations were designed to identify possible functions for mesosomes in Neurospora. Two areas were explored, both suggested by the morphological resemblance

of Neurospora mesosomes to those of bacteria. The possibility of mesosomes having respiratory activity was investigated histochemically, using succinic dehydrogenase as a marker, and employing tetrazolium salts or ferricyanide as electron acceptors. Also, cells of Neurospora were fractionated, and the fractions assayed biochemically for succinic dehydrogenase activity to determine if extra-mitochondrial activity was present. These investigations indicated that respiratory (succinic dehydrogenase) activity is associated with the plasma membrane, mesosomal membranes, as well as mitochondrial membranes in Neurospora.

The possibility of mesosomes being associated with mitochondrial DNA replication in Neurospora was also investigated. These investigations required radioactive labelling of mitochondrial DNA specifically to high levels of activity. This was found to be very difficult, both in vivo and in vitro. However, as a consequence of these studies, much information about factors influencing incorporation of DNA precursors (^{3}H-deoxythymidine triphosphate) was obtained. Also, the experimental results obtained suggest that mitochondrial DNA is associated with some membrane, probably the mitochondrial membrane.

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INTRODUCTION

Mesosome-like structures are seen in the mitochondria and in the cytoplasm in electron micrographs of mycelia of Neurospora crassa. These structures were simultaneously reported in wild type Neurospora by Beck and Greenawalt (1) and in the poky mutant of Neurospora by Malhotra (2). Beck and Greenawalt observed these mesosomes most frequently in germinating conidia and also observed that a high concentration of glucose in the growth medium increased their frequency. From these data, they proposed a biogenetic function for these structures. Malhotra compared these mesosomes to bacterial mesosomes, and suggested the possibility that they might have analogous functions. This was indicated by the observation of these structures in poky Neurospora which is a respiratory deficient mutant, which might require supplementary respiratory organelles. This mutation is maternally inherited and is carried by cytoplasmic determinants (3), probably by mitochondrial DNA. The mutation affects the amount of binding of various components that are present within the mitochondria, and therefore the growth rate. However, the identification by Beck and Greenawalt of mesosomes in wild type Neurospora made it seem unlikely that these structures

serve a respiratory function.

Mesosome-like structures in mitochondria have been reported from a variety of embryonic vertebrate tissues by Ferreira and Ferreira (4), using standard methods of electron microscopy, including fixation in glutaraldehyde and OsO_4.

The possibility that these mesosomes, and the mesosomes of Neurospora, are artifacts of fixation was suggested by the work of Curgy (5), who reported similar structures in embryonic chick hepatocytes. He presented evidence to show that these structures could be induced by double fixation (fixation in glutaraldehyde followed by OsO_4).

The investigations reported in this paper had two aims. First, to examine the reality and morphology of mesosomes under various conditions; and second, to attempt to establish a function for these mesosomes. Investigations of the general morphology of mesosomes in Neurospora were carried out to determine characteristics of mesosomes. These investigations included examination of Neurospora at various growth stages, the effect of various culture conditions on the mesosomes, and the effect of differing techniques of fixation and embedding.

Investigations of possible functions for mesosomes were suggested by their morphological similarity to bacterial

mesosomes. Bacterial mesosomes are membranous infoldings of the plasma (limiting) membrane and have been observed in many types of bacteria. They have been shown to be associated with DNA replication (6), and histochemical tests have demonstrated respiratory activity in these membranes (7).

Attempts to show a respiratory function in Neurospora mesosomes were not necessarily aimed at demonstrating a specific respiratory function for these organelles, but rather to use respiratory enzymes as markers for mitochondrial percursor materials. A role in mitochondrial biogenesis for mesosomes was considered in which mesosomes would provide a method of transport of mitochondrial material from the site(s) of synthesis to the mitochondria. Demonstration of mitochondrial components associated with mesosomal membranes could serve as evidence for a mesosomal role in mitochondrial biogenesis. Transportation of proteins from microsomes to mitochondria has been demonstrated in rat liver cells (8).

Investigations of a possible role for Neurospora mesosomes in mitochondrial DNA replication were suggested by the analogous structures in bacteria, and by the observed close spatial association between some mesosomes and mitochondria in Neurospora. Also, similar structures have been observed in yeast by Yotsuyanagi (9). He proposed that these structures may have a role in replication of mitochondrial DNA in yeast.

Two approaches to this problem were considered, one employing electron microscopic autoradiography, and the other using a detergent fractionation method introduced by Tremblay et al. (10). Considerable difficulty was encountered in the application of these techniques to Neurospora, due to the requirement of both techniques for specifically labelled mitochondrial DNA. It has been shown (11, 12, 13) that DNA of Neurospora cannot be specifically labelled in vivo by the conventional technique of supplying exogenous labelled thymidine. Neurospora lacks a thymidine kinase activity, and specific labelling does not result. Exogenous thymidine is metabolized through thymine, 5-hydroxymethyluracil, 5-formyluracil, to uracil and then is used in pyrimidine synthesis (13). If the supplied thymidine is labelled in the ring, then the label is recovered in RNA and DNA in equal amounts. Also, in vivo labelling by use of radioactive thymidine triphosphate has been shown to be non-specific and highly inefficient, apparently due to the permeability barrier of the plasma membrane (12). Because of these problems, attempts were made to label mitochondrial DNA in vitro, and investigations concerning mitochondrial DNA and mesosomes were limited to isolated mitochondria.

MATERIALS AND METHODS

I. Morphological Studies

Wild type Neurospora crassa (strain 25a) was used for all the studies reported in this paper. The culture was maintained on slants of Horowitz (14) complete medium (Fig. 1), and conidia were obtained in large quantities when required by growing the organism in 500-1000 ml flasks containing the same medium. An increased number of conidia were obtained by coating the sides of the flask with the medium just before solidification. Conidia were harvested by vigorous shaking with sterile water. The resulting suspension was filtered through glass wool and adjusted to the desired concentration by centrifugation or by filtration through glass fiber filters (Reeve-Angel) on a Millipore apparatus. The concentration of conidia was determined by the optical density of the suspension at 600 mu (Fig. 2).

Mycelia were obtained by growth of conidia in Vogel's minimal liquid medium (Fig. 3) by the method described by Eakin (15, 16). The medium was made as a 50x concentrate, diluted and autoclaved or Millipore filtered prior to use, and the appropriate carbon source (usually 2 % sucrose) added. Cultures were usually inoculated with between 10^6

10^{10} conidia, depending on the growth rate and growth stage desired. Young cells were harvested by filtration through glass fiber filters on Millipore apparatus. Older cultures were more easily harvested by filtration through cheese cloth or flannel. After harvesting, the cells were washed in water several times and pressed until most of the water was removed. Small amounts of mycelia for fixation could easily be obtained at any growth stage by filtration through glass fiber filters. The mats thus formed could be cut into small pieces which remained intact throughout processing for electron microscopy.

Growth of mycelia during culturing could be monitored by observing the increase in optical density at 600 nm of the culture, by determining the dry mass of samples of the culture, or indirectly by monitoring the 0_2 uptake of the culture. Observation of the optical density increase could only be used at early stages of growth, as the elongating cells became tangled into clumps and prevented meaningful optical density determinations.

Fixations of cells for electron microscopy was usually accomplished at room temperature with 3% glutaraldehyde in 0.1 M PO_4 buffer (pH 6.8) for 1 to 2 hours. The fixed cells were then washed with three changes of buffer over an hour, and then post-fixed in unbuffered 2 % OsO_4 for 2 to 4 hours at room temperature. Occasionally one or the other of the fixatives was omitted for experimental reasons. Dehydration

and embedding were accomplished by replacing OsO_4 directly with 98.5 % ethanol. The ethanol was changed 3 times over ½ hour and then substituted by 100 % propylene oxide. After 3 changes of propylene oxide in an hour, a mixture of equal volumes propylene oxide and complete embedding mixture (Epon or Araldite) was substituted for 100 % propylene oxide. The propylene oxide in this mixture was allowed to evaporate off for 24 hours. The tissue was then removed from this mixture, placed in embedding molds, fresh embedding mixture was added, and polymerization was carried out at 60° C for 24 hours.

Glycol methacrylate was occasionally used as an embedding medium because of its water miscibility, thereby avoiding the necessity of dehydrating with ethanol and propylene oxide. When this procedure was employed, the method described by Leduc and Bernhard (17) was employed.

Thin sections were cut on a Porter-Blum MT-2 or a Riechart Om U2 ultramicrotome. Electron microscopy was performed with a Philips EM 200 or EM 300 equipped with a goniometer stage. Sections embedded in epoxy resins were stained for 30 to 60 minutes at room temperature in 2 % aqueous uranyl acetate, rinsed, and stained 3 to 5 minutes in 0.2 % aqueous lead citrate. Sometimes lead citrate was used alone. Sections embedded in glycol methacrylate were stained in 2 % aqueous uranyl acetate for 30 minutes at

60°C, rinsed, and stained 5 minutes at room temperature in 0.2 % lead citrate.

FIGURE 1

Horowitz complete solid medium for culturing Neurospora

HOROWITZ COMPLETE SOLID MEDIUM

To 800 ml H_2O, add

K_2 Tartrate	5.0 g
$NaNO_3$	4.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g
NaCl	0.1 g
KH_2PO_4	1.0 g
$CaCl_2$	0.1 g
Casamino acids	0.25 g
Yeast extract	5.0 g
Malt extract	5.0 g
Glycerol	20.0 g (16 ml)
Agar	15.0 g

plus H_2O to 1000 ml final volume. This must be autoclaved immediately.

FIGURE 2

Graph used in determinations of conidial concentration in aqueous suspensions by optical density measurements. From R. T. Eakin (15).

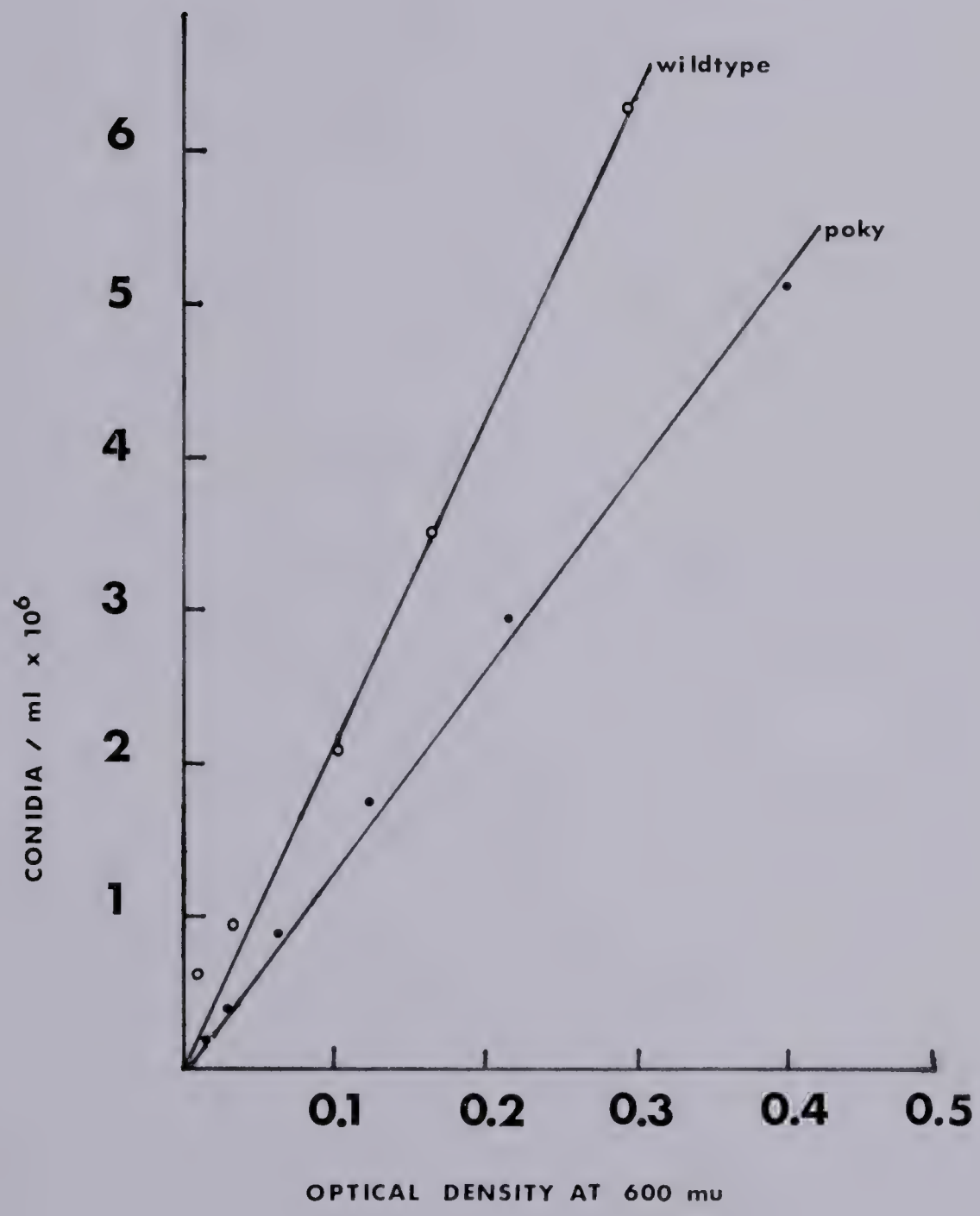

wildtype
poky
6
5
4
3
2
1
CONIDIA / ml x 10^6
0.1
0.2
0.3
0.4
0.5
OPTICAL DENSITY AT 600 mu

FIGURE 3

Vogel's minimal liquid medium for Neurospora, 50x concentrate. The diluted medium is used for culturing large amounts of mycelia.

VOGEL'S MINIMAL MEDIUM FOR NEUROSPORA, 50X CONCENTRATE

To 1500 ml H_2O, add

$CaCl_2$	7.4	g
$MgSO_4 \cdot 7H_2O$	20.0	g
NH_4NO_3	200.0	g
Na citrate	250.0	g
KH_2PO_4	500.0	g
Biotin solution (0.1 mg/ml)	5.0	ml
$CHCl_3$	5.0	ml
Trace element solution (see below)	10.0	ml

Dilute 1:50 with H_2O, make to 2 % sucrose, autoclave.

Do not autoclave concentrated salt solution.

TRACE ELEMENT SOLUTION:

To 95 ml H_2O, add

Citric acid	5.0	g
$ZnSO_4 \cdot 7H_2O$	5.0	g
Fe(II) $(NH_4)_2SO_4 \cdot 6\ H_2O$	1.0	g
$CuSO_4 \cdot 5H_2O$	0.25	g
$MnSO_4 \cdot 1H_2O$	0.05	g
H_3BO_3 (anhydrous)	0.05	g
$Na_2MoO_4 \cdot 2\ H_2O$	0.05	g
$CHCl_3$	1.0	ml

II. Functional Studies

A. Respiratory Activity

Investigations into possible respiratory function of mesosomes were made by histochemical and biochemical techniques. Succinic dehydrogenase activity (SDH) was used as a typical mitochondrial component throughout these investigations.

Two histochemical techniques were employed in attempts to locate SDH activity in electron micrographs of Neurospora cells. One method employed TNBT (tetranitro blue tetrazolium chloride) as described by Leene and van Iterson (7). TNBT is a tetrazolium salt, containing two positively charged nitrogen atoms which can function as electron acceptors. In the presence of succinate and the enzyme, succinic dehydrogenase, electrons are transferred from succinate to the charged nitrogens in the tetrazolium salt. These electrons do not necessarily come directly from SDH, they are most likely accepted from some level several steps removed from SDH in the electron transport system (18). However, SDH activity is required to initiate the flow of electrons from succinate. Upon acceptance of two electrons, the ditetrazolium molecule is reduced to a colored, water insoluble diformazan which precipitates out of a solution at the site of reaction.

The incubation medium for treatment of cells with TNBT consisted of 0.05 % TNBT and 0.25 M Na succinate in 0.1

M PO_4 buffer at pH 7.5. Control incubation media were either without succinate or had Na malonate (a competitive inhibitor) added to excess.

Several problems were encountered in the application of this technique to electron microscopy of Neurospora. While the staining results were intense and reproducible at the light microscope level, difficulty was experienced in recognizing the reaction product in sections observed in the electron microscope. Also, it was found difficult to reproduce experimental results. These problems were thought to arise from the low electron density of the reaction product in the sections.

Because of these problems encountered in using these techniques, experiments were performed in an attempt to determine the amount of reduced TNBT necessary to be reproducibly observed in the electron microscope. Reduced tetrazolium was dissolved in amyl acetate in varying concentrations and applied directly to formvar coated grids and allowed to dry. The electron density of the dried formazan was measured at several places on the grid in the Philips EM 300 by use of the exposure meter, and by photographing the film on plates and recording the resulting optical density on a Joyce-Loebl scanning microdensitometer. The results were expressed as the difference in optical density between the background (formvar film + no formazan) and the film (formvar film + formazan), and were plotted

against the concentration of the formazan applied to the grid.

The second, and more extensively employed histochemical method was the potassium ferricyanide-copper sulfate technique introduced by Ogawa et al. (19). This method employs ferricyanide as an electron acceptor for SDH activity. As with the tetrazoliums, it is not certain where in the electron transport system that ferricyanide is reduced. However, SDH activity is required for the reduction of ferricyanide in the presence of succinate (19). The reduced ferricyanide combines rapidly with Cu^{++} ions present in the medium, and precipitates out at the site of reaction as insoluble, electron dense, copper ferrocyanide. This reaction product is difficult to discern in the light microscope, but is easily recognizable in sections in the electron microscope.

The ferricyanide medium contained in each ml: 100 umoles PO_4, pH 7.5; 18 umoles Na succinate; 3 umoles Na citrate; 3 umoles $CuSO_4$; and 0.5 umoles $K_3Fe(CN)_6$. Control media were either without succinate or had malonate added, or both.

Cells subjected to the histochemical procedures were either freshly harvested or prefixed for 10 minutes in cold 3 % formaldehyde or 3 % glutaraldehyde in 0.1 M PO_4, pH 7.3. They were incubated in the reaction mixture for 30 minutes

at 27°C with constant agitation. After incubation, the cells were collected by filtration, washed, and fixed in 3 % glutaraldehyde at room temperature for up to 12 hours. In some cases, the cells were post-fixed in 2 % OsO_4 in 0.1 M PO_4, pH 7.3 for 3 hours. The fixed cells were embedded as previously described, and thin sections were examined without staining in the Philips EM 200 or EM 300.

Possible extra-mitochondrial SDH activity was assayed for in subcellular fractions of Neurospora. These fractions were obtained by homogenizing freshly harvested mycelia in an equal volume of isolation medium (0.25 M sucrose; 0.1 % BSA; 0.003 M EDTA; 0.05 M tris-HCl, pH 7.5) by grinding with sand in a mortor. The mycelia were ground until the homogenate was free of lumps and had the consistency of thick pea soup. Mitochondrial and microsomal fractions were prepared from this homogenate (as illustrated in Fig. 4) by differential centrifugation. All operations were carried out at 0° - 3° C.

SDH activity of these fractions was assayed by the method described by Green et al. (20) at 37°C in a solution consisting of in each ml: 100 umoles PO_4, pH 6.8, 500 ug BSA; 2.0 umoles NaCN; 20 ug 2,6-dichlorophenolindolphenol; 10 umoles Na succinate; and 100 ug mitochondrial protein or 500 ug microsomal protein. Succinate was added separately to initiate the reaction, and the reaction was followed by

observing the reduction in optical density at 600 mu of the assay medium against a blank identical to the sample except succinate was deleted. SDH units were defined as reduction in optical density at 600 mu per minute per milligram protein at 37°C. Protein concentration was determined by the method of Lowry et al. (21).

Cross contamination of the microsomal fraction by mitochondrial material was checked by electron microscopical examination of negatively stained samples of the fractions, and by assaying for oligomycin sensitive (F_1) ATPase activity. F_1 ATPase activity of the fractions was assayed at 37°C in a medium containing in each ml: 250 umoles sucrose; 10 umoles $MgCl_2$; 50 umoles tris-HCl, pH 7.5; 1.0 umole ATP; 10 ug oligomycin; and 0.5 mg mitochondrial protein or 1.0 mg microsomal protein. The reaction was followed by sampling at intervals and determining the amount of Pi released by the method of Taussky and Shorr (22). F_1 ATPase units were defined as ug Pi released per minute per milligram protein at 37°C.

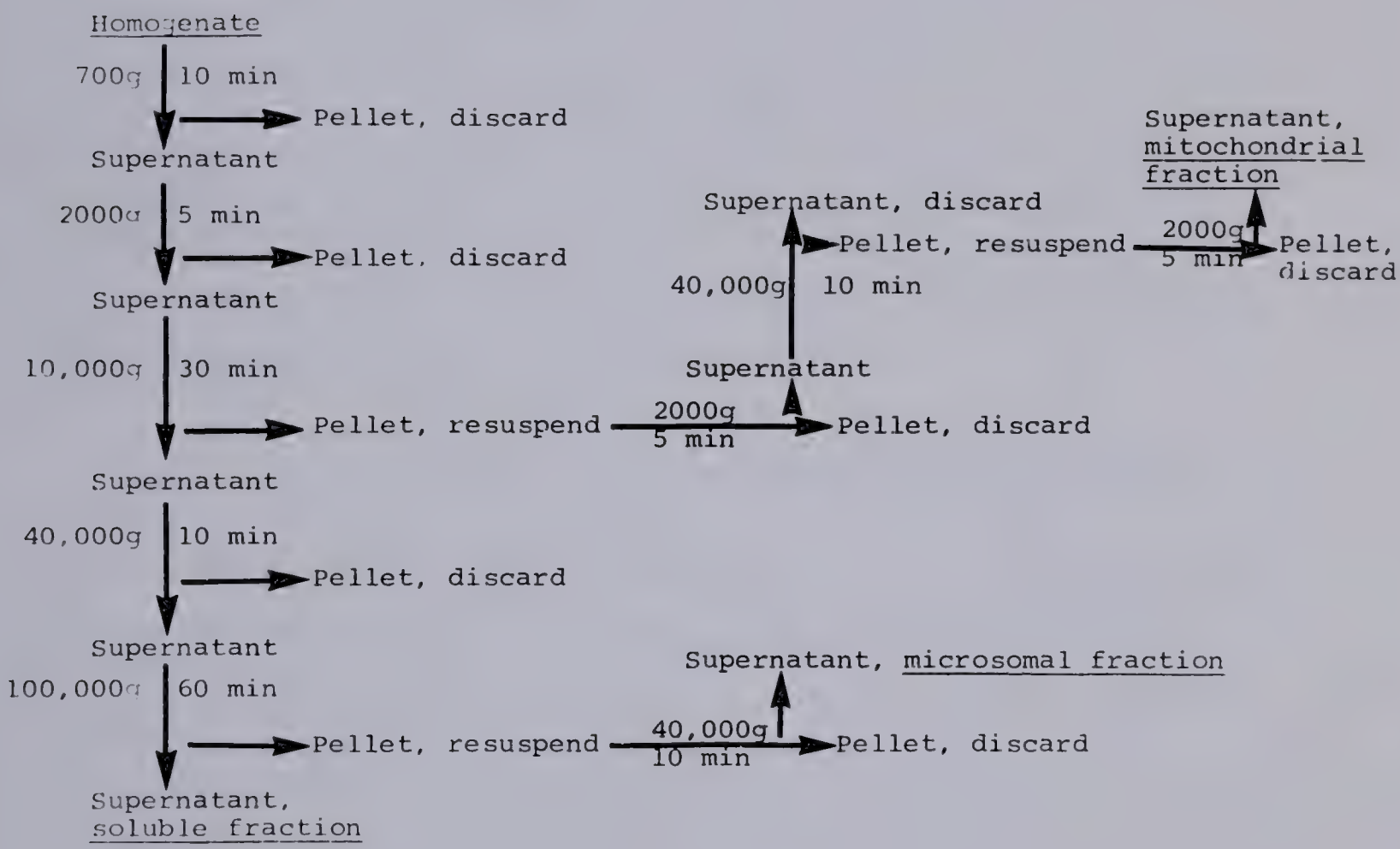
Parallel studies to those just described on SDH activity in Neurospora were performed on rat liver cells both in situ histochemically, and in isolated fractions biochemically. For histochemical demonstration of SDH activity, the liver was immediately (within 60 seconds) removed from a rat killed by neck dislocation, and placed

into a chilled medium consisting of 0.25 M sucrose; 0.1 % BSA, 0.003 M EDTA, and 0.05 M tris-HCl at pH 7.5. The liver tissue was cut into rectangular pieces about 1 x 3 mm, affixed to filter paper discs with warm agar, and chopped into approximately 125 u slices by a Smith-Farquar tissue chopper. These slices were placed into a ferricyanide medium identical to that used for demonstration of SDH activity in Neurospora, incubated 30 minutes at 37°C, washed, doubly fixed in glutaraldehyde and OsO_4, and processed routinely for electron microscopy.

Subcellular fractions of rat liver were obtained by the method described by Mahler and Cordes (23). These fractions were assayed as previously described for Neurospora for both SDH activity and F_1 ATPase activity. Mitochondrial, microsomal, rough microsomal, and smooth microsomal fractions were assayed.

FIGURE 4

Isolation method for obtaining mitochondrial, microsomal, and soluble fractions from Neurospora. The mycelia were homogenized as described in the text, and then differentially centrifuged as indicated.


Homogenate
700g
10 min
Pellet, discard
Supernatant
2000g
5 min
Pellet, discard
Supernatant
10,000g
30 min
Pellet, resuspend
2000g
5 min
Pellet, discard
Supernatant
40,000g
10 min
Supernatant, discard
Pellet, resuspend
2000g
5 min
Pellet, discard
Supernatant, mitochondrial fraction
Supernatant
40,000g
10 min
Pellet, discard
Supernatant
100,000g
60 min
Pellet, resuspend
40,000g
10 min
Pellet, discard
Supernatant, microsomal fraction
Supernatant, soluble fraction

B. Mitochondrial DNA Replication

Investigations of a possible role in mitochondrial DNA (mDNA) replication for mesosomes in Neurospora were limited to in vitro studies for reasons given in the introduction. Because in vitro incorporation of radioactive nucleoside triphosphates into mDNA had been demonstrated in several organisms (24, 25, 26), including yeast (27), this activity was investigated in Neurospora. It was hoped that this activity could be used to provide radioactive mDNA in Neurospora for use in further experimentation. Tritiated thymidine triphosphate (^{3}H-TTP) was used in this investigation as a radioactive DNA precursor.

Mitochondria for these experiments were isolated by the method essentially described by Luck (28). The isolation medium consisted of 0.44 M sucrose; 0.1 % BSA; 0.003 EDTA; and 0.05 M tris-HCl, pH 7.5. Mitochondrial fractions prepared in this medium by differential centrifugation were purified on 0.9 M to 1.9 M sucrose density gradients by centrifugation at 140,000g for 90 minutes. The mitochondrial bands formed were removed by pipet and diluted with a storage medium (0.25 M sucrose; 0.1 % BSA; 10 uM B-mercaptoethanol; 0.05 M tris-HCl, pH 7.5) to approximately 0.8 M sucrose. The mitochondria were then pelleted and resuspended in a small volume of storage medium. Protein concentration was determined by Lowry's technique (21), using crystalline

BSA as a standard, and adjusted to 2 mg/ml. The condition and purity of the mitochondrial fractions were checked by examination of negatively stained samples (2 % potassium phosphotungstate) in the electron microscope.

Incorporation of ^{3}H-TTP into an acid insoluble product by isolated mitochondria of Neurospora was assayed in a medium modified from Wintersberger (27) for assay of mitochondrial DNA polymerase activity in yeast. This medium contained in each ml: 50 umoles tris-HCl, pH 7.5; 10 umoles $MgCl_2$; 1.0 umole each of dATP, dCTP, dGTP (from Sigma Chemical Co.); 2.5 uC ^{3}H-TTP (9.8 C/mmole, from Schwartz Bio-research); 200 umoles sucrose; 3.0 mumoles B-mercaptoethanol; and 1.0 mg mitochondrial protein. The incubation temperature was usually 27^{o}C. Incorporation was initiated by addition of the mitochondrial protein, and the amount and rate of incorporation was determined by withdrawing 0.05 ml samples at intervals. These samples were applied to 1 inch filter paper discs or glass fiber filters previously marked with pencil to facilitate sorting after washing. After the sample had been completely absorbed by the filter (about 5 seconds), the filters were immediately dropped into a large volume of cold 10 % TCA (trichloroacetic acid) and processed by the method described by Bollum (29). The method involved washing the filters twice with a large volume (50 ml per filter) of cold 5 % TCA, followed by two similar washings with cold 95 % ethanol, followed by two similar washings with ethyl ether. The filters were then

allowed to dry, sorted, placed into liquid scintillation counting vials with 10 ml of a toluene based scintillant, and counted in a Nuclear Chicago Mark I liquid scintillation counter. Counting efficiency was determined by the channel ratios method. The amount of ^{3}H-TTP incorporated was calculated from the acid insoluble dpm and the specific activity of the ^{3}H-TTP used, as determined by the supplier.

Conversion of ^{3}H-TTP to ^{3}H-TMP (tritiated thymidine monophosphate) during the incorporation of ^{3}H-TTP was detected by sampling a portion of the assay mixture at intervals into cold 10 % TCA. After 5 minutes at 0^{o}C, the samples were centrifuged and 5.0 ul of the supernatant was spotted onto a thin layer silica gel chromatogram (Eastman Kodak). 5.0 ul of 1.0 mM TTP and 5.0 ul of 1.0 mM TMP were added to the spots as carriers, and the chromatograms were run in a solvent system consisting of 700 ml of 98.5 % ethanol, 300 ml of 1.0 M ammonium acetate and 0.33 M EDTA, and 100 ml of glacial acetic acid. The pH of the solvent was 3.8. After 20 hours, the chromatogram was examined with ultraviolet light, and the spots containing TTP and TMP were cut out. The radioactivity of the spots was determined by placing them directly into counting vials with a toluene based scintillant, and counting in a liquid scintillation counter.

Possible mitochondrial nuclease activity was assayed

by measuring the conversion of ^{3}H labelled DNA (from E. coli) to products soluble in 2 % perchloric acid. The ^{3}H labelled DNA was prepared by the addition of ^{3}H-thymine (1.0 mC/0.008 mg, New England Nuclear) to a culture of E. coli, strain K 12 thy -, requiring 2 ug thymine per ml, and grown in a synthetic medium. DNA was isolated by the method of Marmur (30), and had a specific activity of 4.2 x 10^{5} cpm per umole. The assay mixture contained in 0.11 ml: 15 nmoles native or denatured ^{3}H-DNA; 1.0 umole $MgCl_2$; 5.0 umoles tris-HCl, pH 7.5; and sufficient enzyme (mitochondrial protein) to convert 5 to 40 % of the substrate to acid soluble products. The incubation temperature was 37°C. After 20 minutes, the assay was terminated by putting 0.1 ml of the mixture into a microcuvette containing 0.1 ml cold perchloric acid, and 0.1 ml of 2.5 mg/ml calf thymus DNA was added as a carrier. The samples were kept 5 minutes at 0°C, and then centrifuged 1 minute in a Beckman microfuge. 0.2 ml of the supernatant was counted in Bray's solution (31). The nuclease activity of the mitochondrial preparation was constant with time and linear with protein concentration in the range given. One unit of activity was defined as that amount catalyzing the production of 1.0 umole of acid soluble nucleotide in 20 minutes.

Tritiated actinomycin D (^{3}H-actD) was also employed as a method of labelling mDNA. Mitochondrial suspensions containing 1 to 2 mg/ml protein were incubated with 0.5 to

1.0 uC ^{3}H-actD (3.38 C/mmole, Schwartz Bio-research) in the absence of Mg^{++} and in the dark at room temperature for 60 minutes. Specificity of ^{3}H-actD binding to mDNA was demonstrated by freezing and thawing such a preparation, dividing it into two aliquots, and incubating one with DNAse. Both aliquots were then washed and layered onto 30 to 60 % sucrose gradients, centrifuged at 140,000g for 90 minutes, and fractionated. The optical density at 280 mu and radioactivity of each fraction was measured (Fig. 5).

Isolation of a membrane-DNA complex from *in vitro* ^{3}H-TTP or ^{3}H-actD treated mitochondria was attempted using the detergent fractionation technique of Tremblay *et al.* (10). This method employs the detergent sodium lauroyl sarcosinate (Sarkosyl, Giegy Chemical Co.). In the presence of Mg^{++}, the detergent crystallizes and the crystals can be banded by sucrose density gradient centrifugation. When this detergent is applied to spheroplasts or other fragile forms of bacteria in the presence of Mg^{++}, 10 to 30 % of the cell membrane, 90 % more of the DNA, and about 75 % of the nascent RNA are found associated with the crystals. The formation of this complex depends on the interaction of membrane components with the detergent crystals, as neither nucleic acids nor ribosomes interact directly with the crystals, but partially purified membranes and phospholipids do (10).

To adapt this technique to *Neurospora* mitochondria,

it was found necessary to replace Mg^{++} with Ca^{++}, as it has been shown (32) that the mitochondrial nuclease activity of Neurospora is activated by Mg^{++}, but is inactive in the presence of Ca^{++}. Ca^{++} was found to cause crystallization of the detergent in the same concentration range as Mg^{++}.

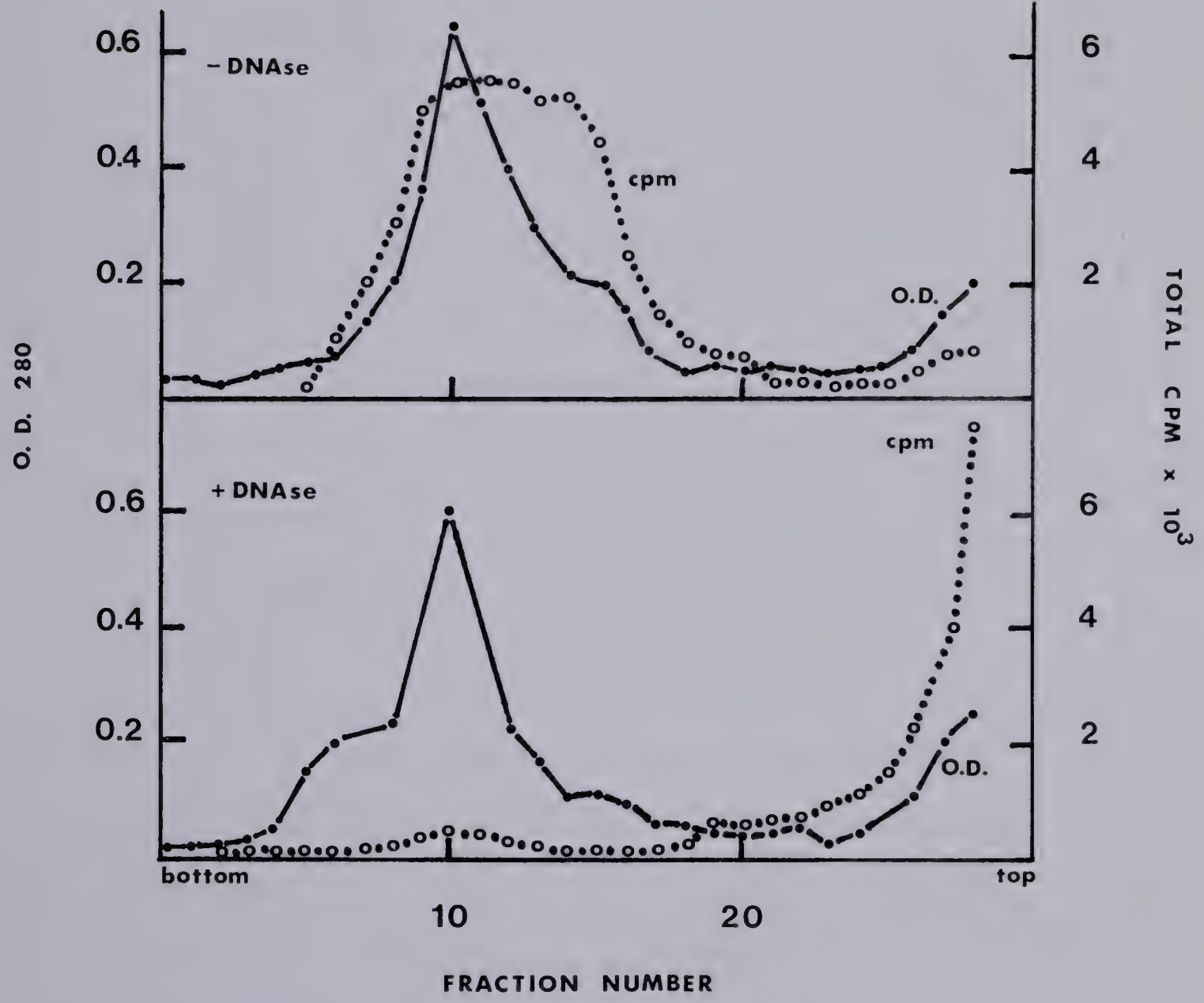
Mitochondria treated with ^{3}H-TTP or ^{3}H-actD were concentrated by centrifugation to 5 to 10 mg protein per ml and layered onto a 30 to 60 % sucrose density gradient containing 0.01 M tris-HCl, pH 7.5; 0.05 M $CaCl_2$' and 0.1 M KCl. Sodium lauroyl sarcosinate was then added to the layered material to a final concentration of 1 to 2 % of the layered volume. The layered material and the detergent were mixed gently so as not to disturb the gradient. Control experiments were performed using identical density gradients, except no detergent was added. The gradients were centrifuged at 140,000g for 90 minutes, and fractionated either by dripping through a hole pierced in the bottom of the centrifuge tube, or by an ISCO density gradient fractionator.

The optical density at 280 and 260 mu, total radioactivity, and acid insoluble radioactivity of each fraction was determined. Optical density determinations were made with a Gilford model 2400 recording spectrophotometer equipped with microcuvettes. Total radioactivity of the samples was determined by adding an aliquot of the sample (usually 0.1 ml) to 10 ml of Bray's solution (31) in a counting vial, and

counting in a scintillation counter. Acid insoluble radioactivity was determined by applying an aliquot of the sample (usually 0.1 ml) to a 1 inch filter paper disc or a glass fiber filter. These filters were then processed by the method of Bollum (29) and counted as previously described.

FIGURE 5

Distribution of optical density at 280 mu (solid line) and radioactivity (cpm, dotted line) after treatment of mitochondria with ^{3}H-actinomycin D and centrifugation on density gradients. The top graph represents the fractionation pattern of mitochondria not treated with DNAse before centrifugation, and the bottom graph represents the results from mitochondria that were treated with DNAse before centrifugation.


0.6
0.4
0.2
– DNAse
cpm
O.D.
6
4
2
O. D. 280
TOTAL CPM x 10^3
+ DNAse
cpm
O.D.
bottom
top
10
20
FRACTION NUMBER

RESULTS

I. Morphology of Mesosomes

Mesosomes of Neurospora appeared as membranous whorls or vesicles which were observed to be continuous with the plasma membrane (Fig. 6). In sections, two apparent types of mesosomes were observed, one closely associated with mitochondria, and another larger and more complex, not associated specifically with mitochondria. Mesosomes were observed most frequently from cells fixed from early growth stages (Fig. 7). Whether this observation is a result of the cells being in a particular developmental stage, or is a result of a statistical effect of sectioning, will be discussed further.

Changes in the carbon source of the culture medium affected the growth rate (Fig. 8) as well as the appearance of mesosomes in the cells. High concentrations of sucrose (7 to 10 %) and dextrose (15 to 25 %) caused an increase in the frequency of observation and the complexity of the large, non-mitochondrial type of mesosome. Mesosomes from cells grown in high concentrations of sucrose or dextrose appeared larger and had a more complex organization. These observations were made only in cells fixed in glutaraldehyde

followed by OsO_4 (Fig. 9).

In sections prepared for electron microscopy, the mesosomal and plasma membranes appeared much more electron opaque than the mitochondrial membrane. This difference in staining was observed when the material was doubly fixed in glutaraldehyde followed by OsO_4, but not when either fixative was used alone.

The method of fixation employed on these cells was found to have a pronounced effect of the morphology of mesosomes, and on the general ultrastructural appearance of the cells. Cells fixed in glutaraldehyde and OsO_4 showed many more mesosomal structures in and near mitochondria (Figs. 10, 11) than did cells fixed only in OsO_4. The membranes of these mitochondrial associated mesosomes could be observed to be closely associated with, but not applied to or penetrating into, mitochondrial membranes (Fig. 12).

Large, complex mesosomal structures not associated with mitochondria were observed frequently in doubly fixed cells (Figs. 13, 14). They were not seen in cells fixed only with OsO_4. These results were not affected by the embedding procedure used (epoxy resins or water soluble glycol methacrylates). In living cells observed at high magnification in a polarizing light microscope, large regular lamellar membranous bodies such as these mesosomes

should be visible by their birefringence. No such birefringent bodies were observed. If these structures existed as membranous vesicles or some other irregular membranous structure in living cells, then they would not be visible under these conditions. In these same preparations, the birefringence of the cell walls of both Neurospora and contaminating bacteria could be seen, as well as that of the nuclear membranes within the Neurospora cells.

Mitochondrial mesosomes also appeared different in cells fixed only in OsO_4. No complex arrays of whorls or tubules, as seen in doubly fixed cells, were observed in cells fixed only in OsO_4. However, simple membranous structures were observed in association with mitochondria in these cells. These structures appeared closely associated with the outer mitochondrial membrane. In some cases, they appeared to link the outer mitochondrial membrane and the plasma membrane (Figs. 15, 16, 17). These membranes connecting the plasma membrane with the outer mitochondrial membrane were observed after fixation in glutaraldehyde or OsO_4, but not after fixation in both.

It was also noted that the plasma membrane appears more complex and irregular in cells fixed only with OsO_4 than in doubly fixed cells. This difference was measured by overlaying with string the plasma membrane in random seg-

ments of cell edges seen in electron micrographs of cells processed identically except for fixation. The length of the cell wall in these segments was also determined, and the ratio of these lengths was used to determine relative differences in the amount of plasma membrane seen in doubly fixed cells and cells fixed only in OsO_4 (Fig. 18). It was found that sections of doubly fixed cells showed a plasma membrane profile with about 40 % less length than OsO_4 fixed cells. Other membranous structures in OsO_4 fixed cells (nuclei, mitochondria) also appeared very irregular and poorly defined when compared to similar structures in cells fixed by glutaraldehyde and OsO_4.

These results imply that growth conditions and, in particular, the method of fixation, affect the organization of membranous structures in Neurospora. The nature of this effect will be discussed further.

FIGURE 6

Electron micrographs showing (1) a large, complex mesosome not associated with mitochondria, and (2) a smaller, simpler mesosome associated with a mitochondrion. Both micrographs are from material fixed in glutaraldehyde and OsO_4, and both show the mesosome continuous with the plasma membrane. The upper micrograph (1) is of an unstained section, the magnification is 62,800. The lower is stained with lead citrate, and the magnification is 120,000.

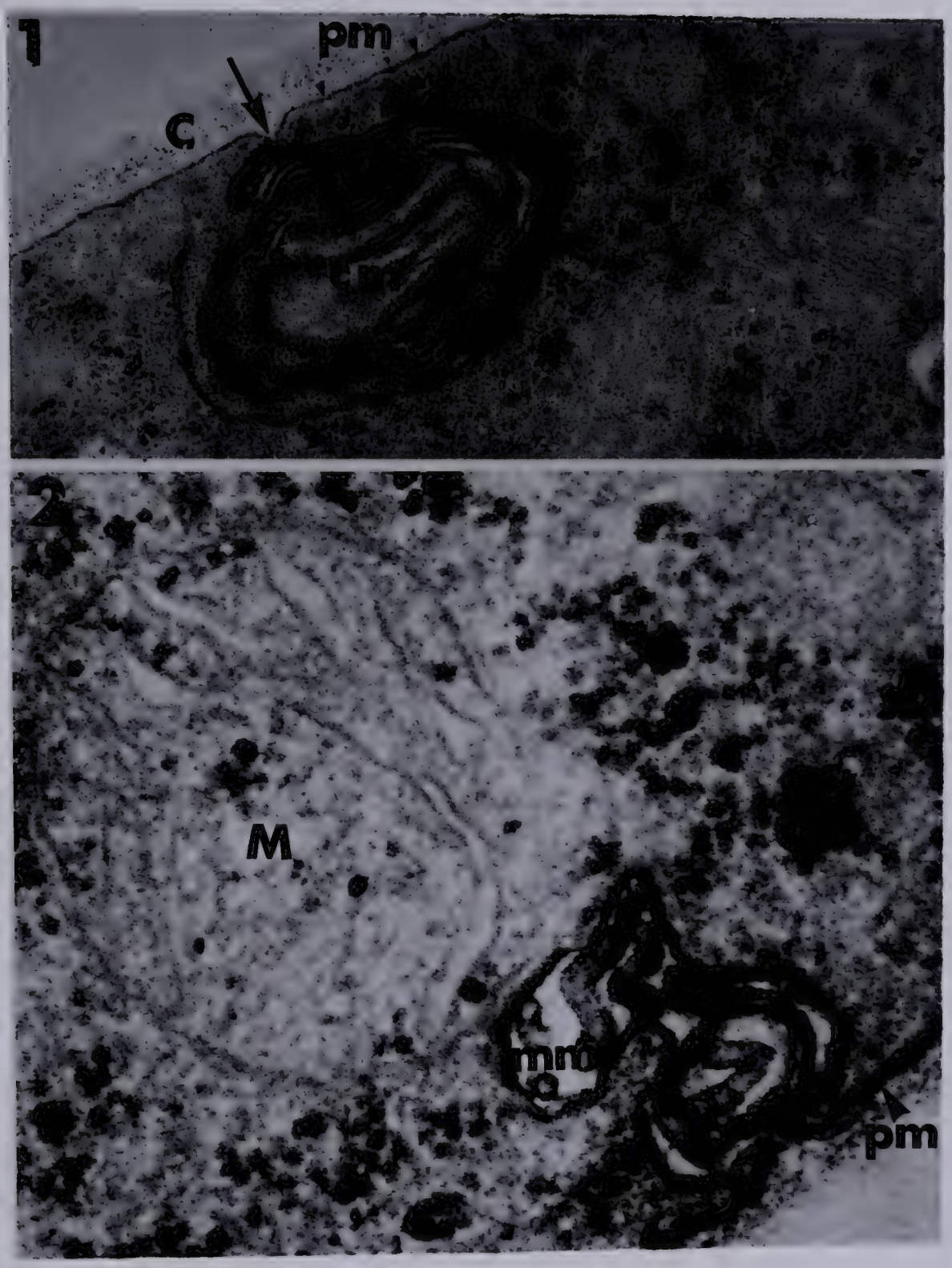

1
pm
c
2
M
mm
pm

FIGURE 7

Graph illustrating the relation of growth of Neurospora in liquid culture (as measured by the optical density of the culture at 600 mu) and the number of mesosomes observed in sections of material fixed at various times from this culture.

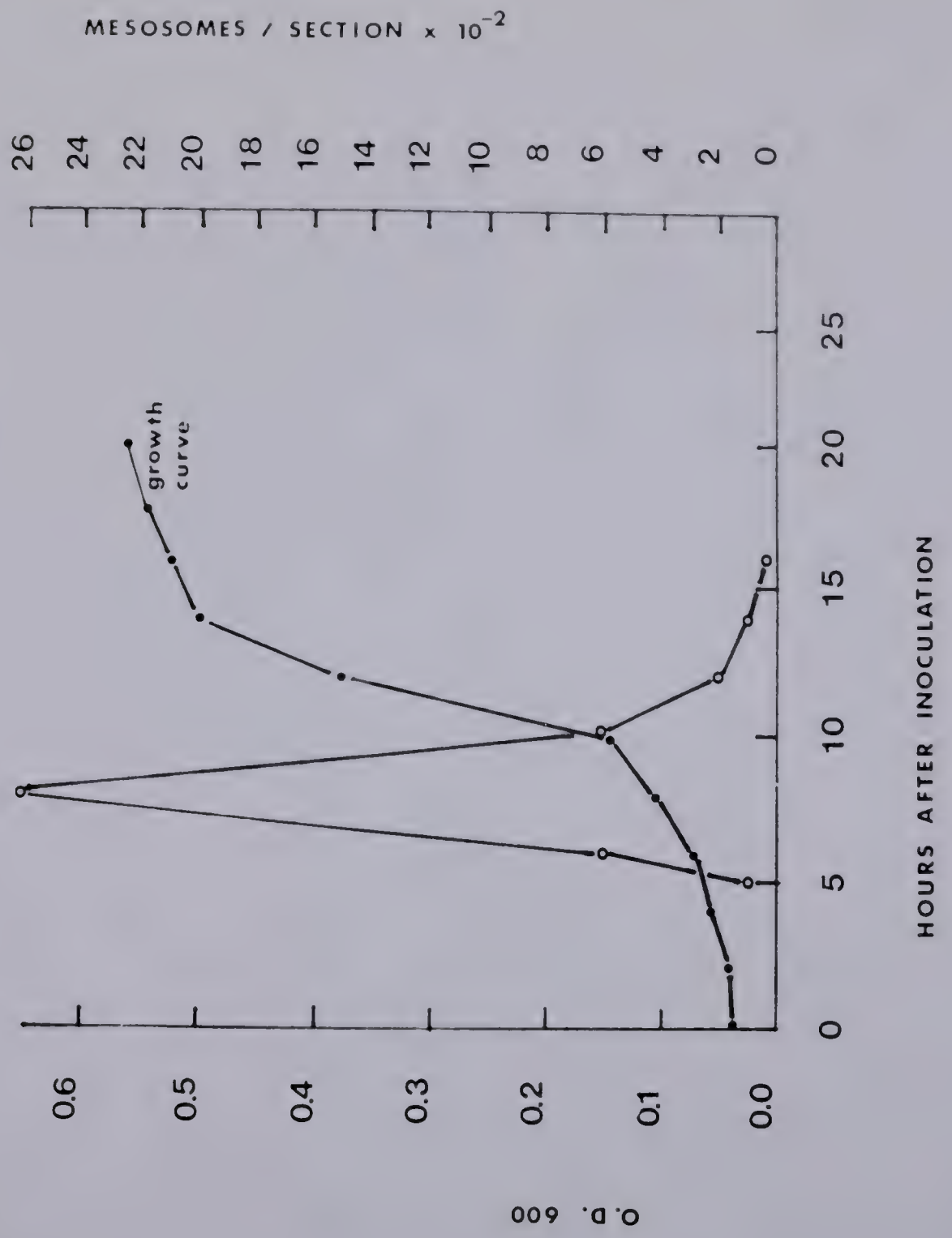

MESOSOMES / SECTION x 10^{-2}
26
24
22
20
18
16
14
12
10
8
6
4
2
0
growth curve
O. D. 600
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
5
10
15
20
25
HOURS AFTER INOCULATION

FIGURE 8

Table showing the amount of Neurospora harvested from Vogel's minimal liquid medium using various carbon sources. The cultures were inoculated with the same number of conidia (5×10^4/ml) and grown under identical conditions for 24 hours. The mycelia were dried over P_2O_5 in a vacuum for 5 days, and then weighed to determine dry mass.

TABLE OF UTILIZATION OF CARBON SOURCES

Carbon Source	Concentration (w/v)	Dry Mass *Neurospora* (g) harvested/liter culture/24 hrs.
sucrose	5 %	4.38
sucrose	10 %	4.20
dextrose	7 %	4.24
dextrose	15 %	2.80
dextrose	20 %	2.45
glycerol	5 %	1.37
glycerol	10 %	1.22
glycerol	15 %	0.83

FIGURE 9

An electron micrograph of a section of a Neurospora cell fixed during the late exponential phase of growth in Vogel's medium containing 10 % sucrose as the carbon source. A large complex mesosome connected with the plasma membrane (arrow) is shown. This material was fixed in glutaraldehyde and OsO_4, and stained with uranyl acetate and lead citrate. The calibration line represents 0.5 micron.

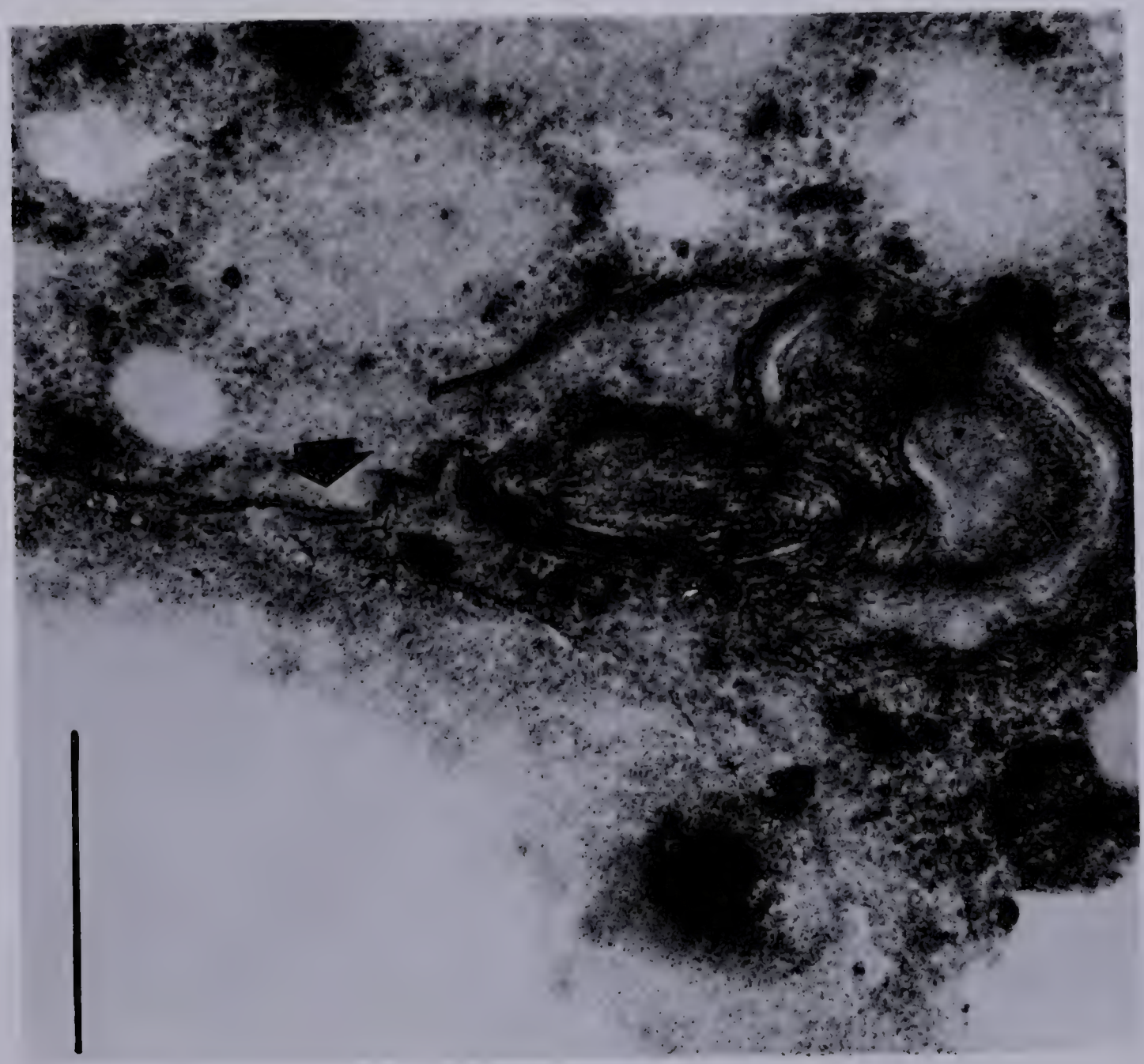


FIGURE 10

An electron micrograph of a doubly fixed Neurospora cell during the early exponential phase of growth. A mesosome apparently within a mitochondrion can be seen (arrow), but this appearance could result from sectioning across a mesosome impressed into a mitochondrion (see Figure 12). A large donut shaped mitochondrion also can be seen (M) surrounding a bit of cytoplasm (cyt). This type of mitochondrion is frequently seen in sections of Neurospora from this stage. Lipid droplets (L) and glycogen granules (G) are also apparent. The calibration line represents 0.5 micron. The material has been stained with lead citrate.

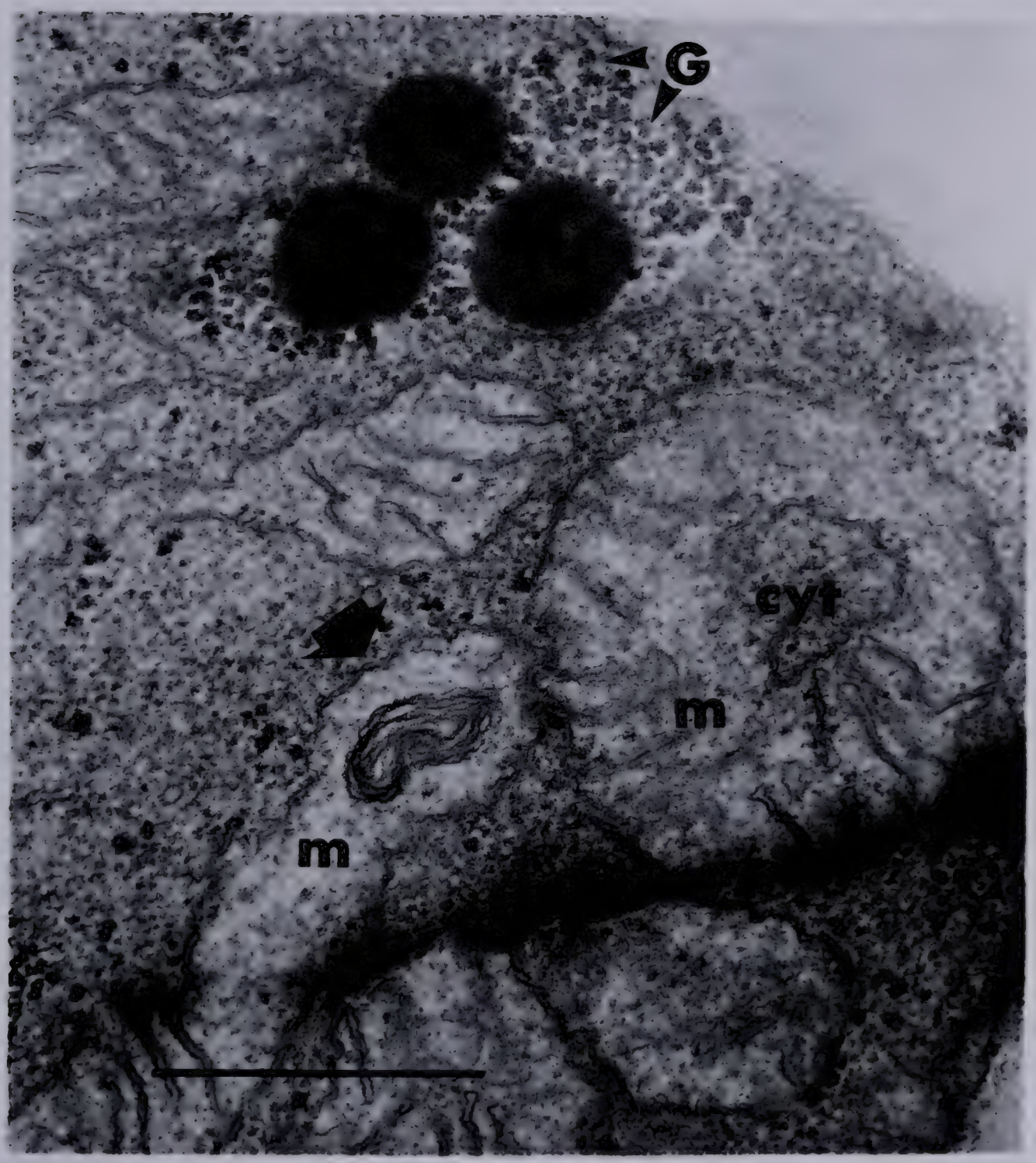

G
cyt
m
m

FIGURE 11

An electron micrograph of Neurospora cell similar to the one seen in Figure 10. The arrow in the center area of the cell points out a mitochondrial mesosome, while the arrow at the lower left indicates a mesosome apparently not associated with a mitochondrion. The calibration line represents 0.5 micron.

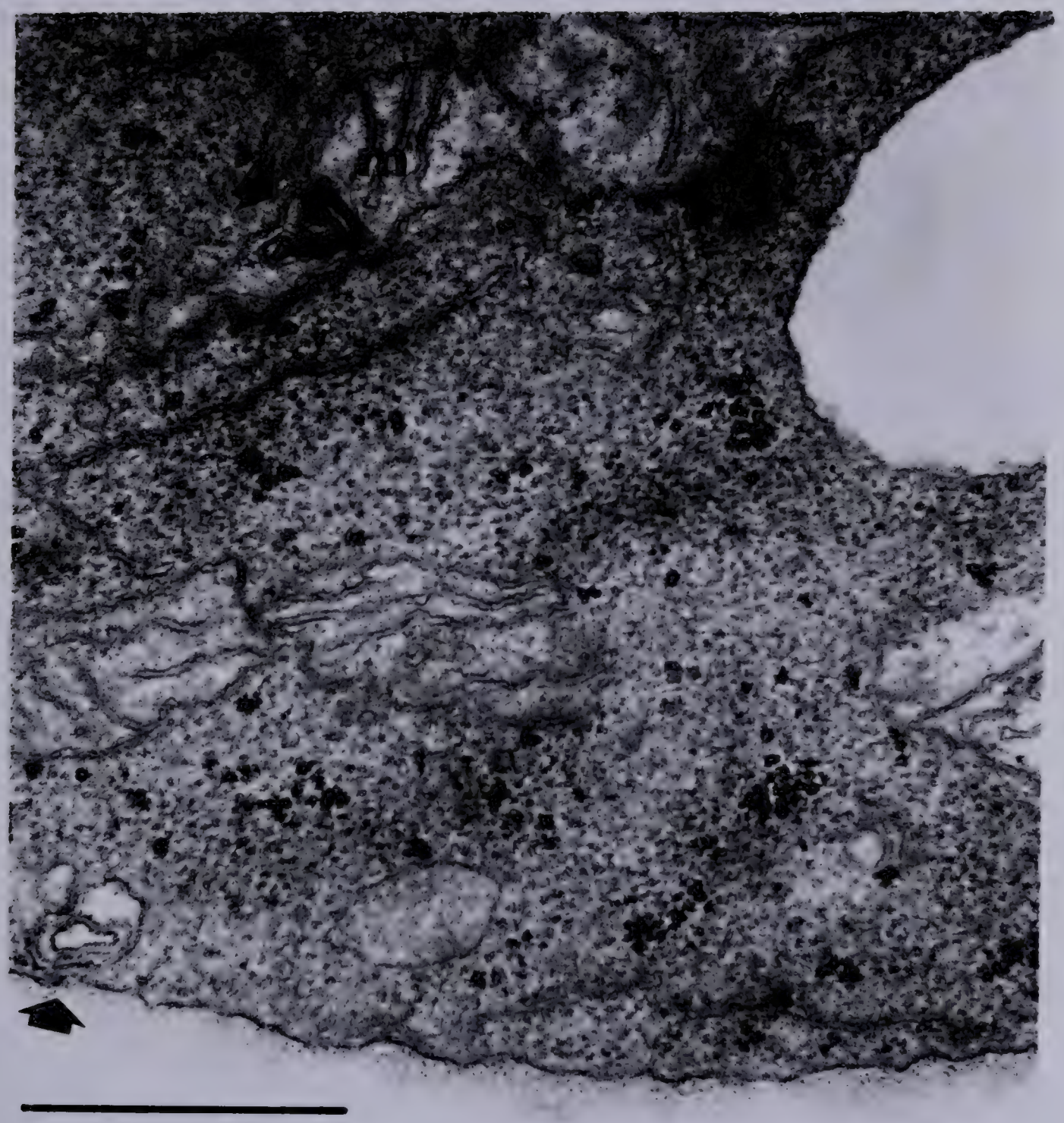
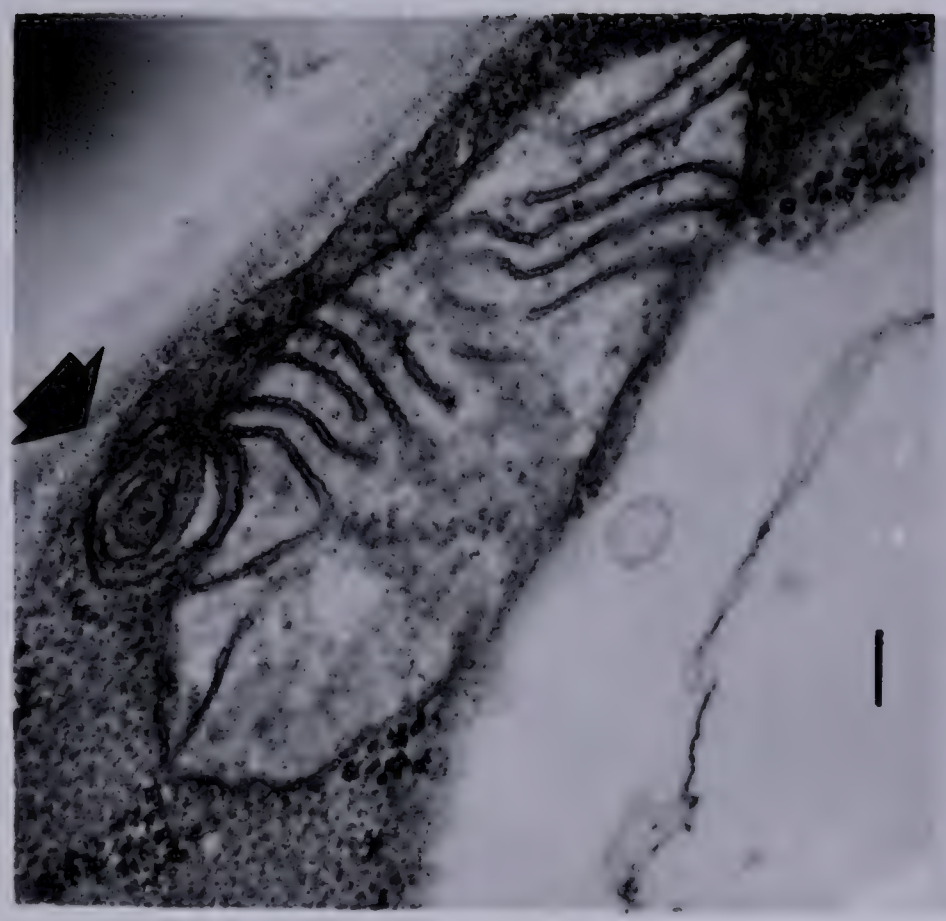


FIGURE 12

An electron micrograph showing close apposition of mesosomes to mitochondria in Neurospora. Note that the mesosomal membranes (arrows) do not contact the mitochondrial membrane. The material was fixed in glutaraldehyde and OsO_4 from a culture in the early exponential growth phase, and stained only with lead. The calibration lines represent 0.1 micron.



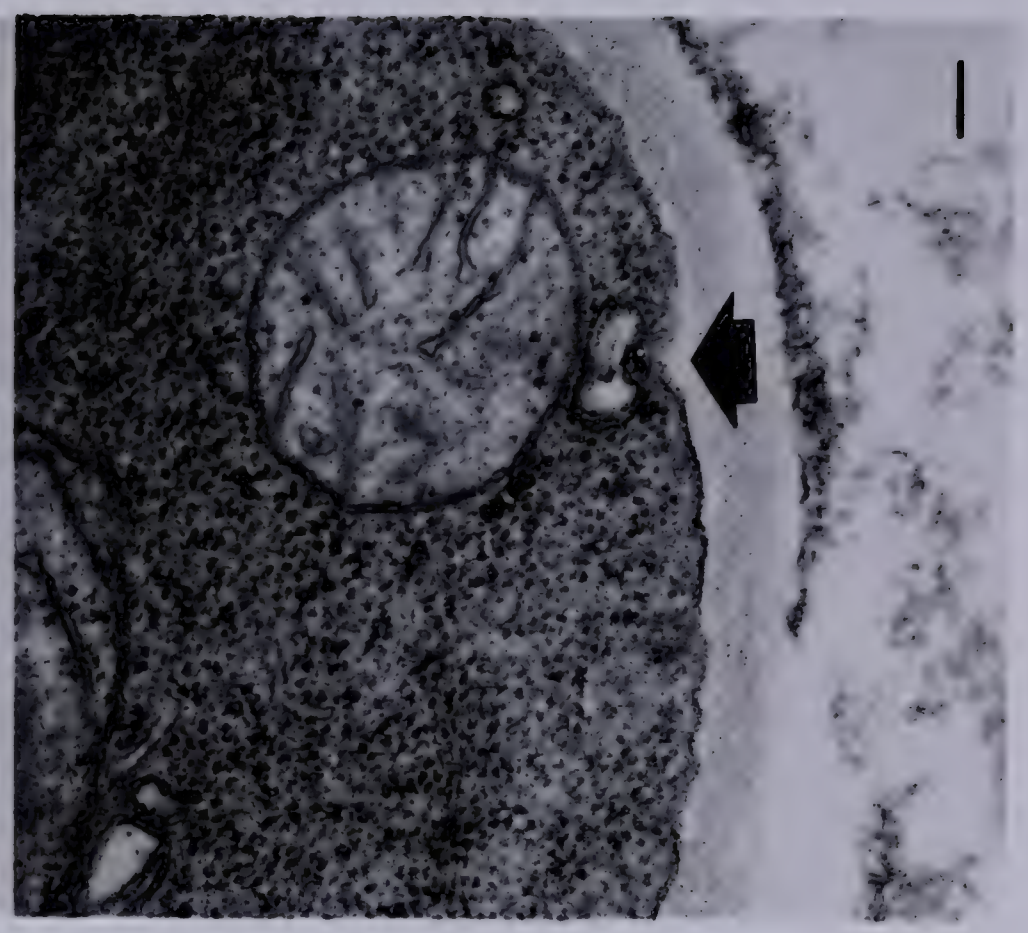


FIGURE 13

An electron micrograph from a cell of Neurospora doubly fixed during the early exponential phase of growth. A mesosome not associated with mitochondria and its connection with the plasma membrane can be seen (arrow). A nucleus (N) can also be seen in this section. This material was stained with lead citrate, and the calibration line represents 0.1 micron.

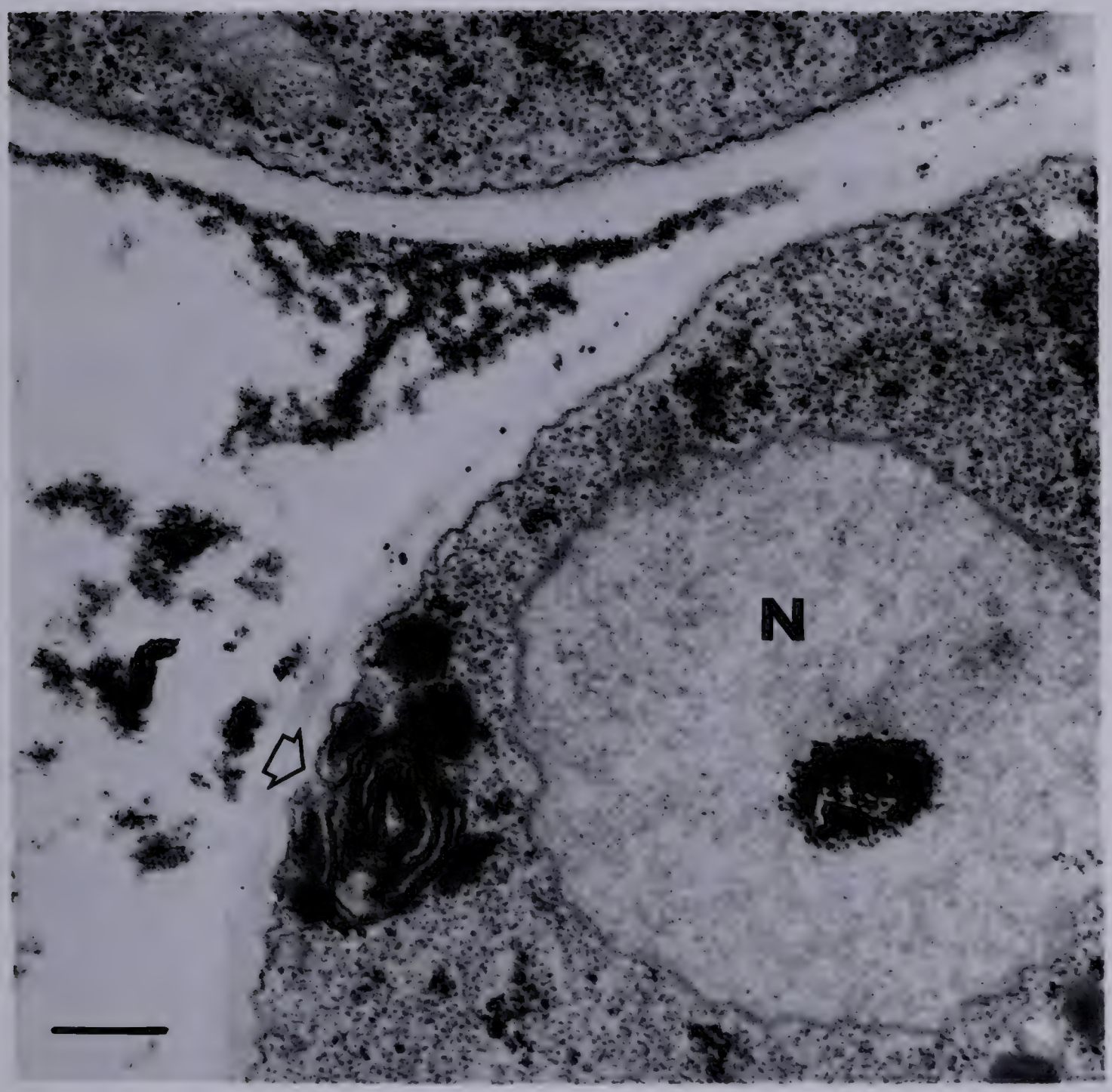

N

FIGURE 14

An electron micrograph illustrating a large complex mesosome not associated with mitochondria. A connection with the plasma membrane can be seen (large arrow) and areas of apparent unit membrane reorganization can be seen within the mesosome (small arrows). This is better illustrated in Figure 37. The material was doubly fixed from a culture in the late exponential phase of growth, and is unstained. The calibration line represents 0.1 micron.

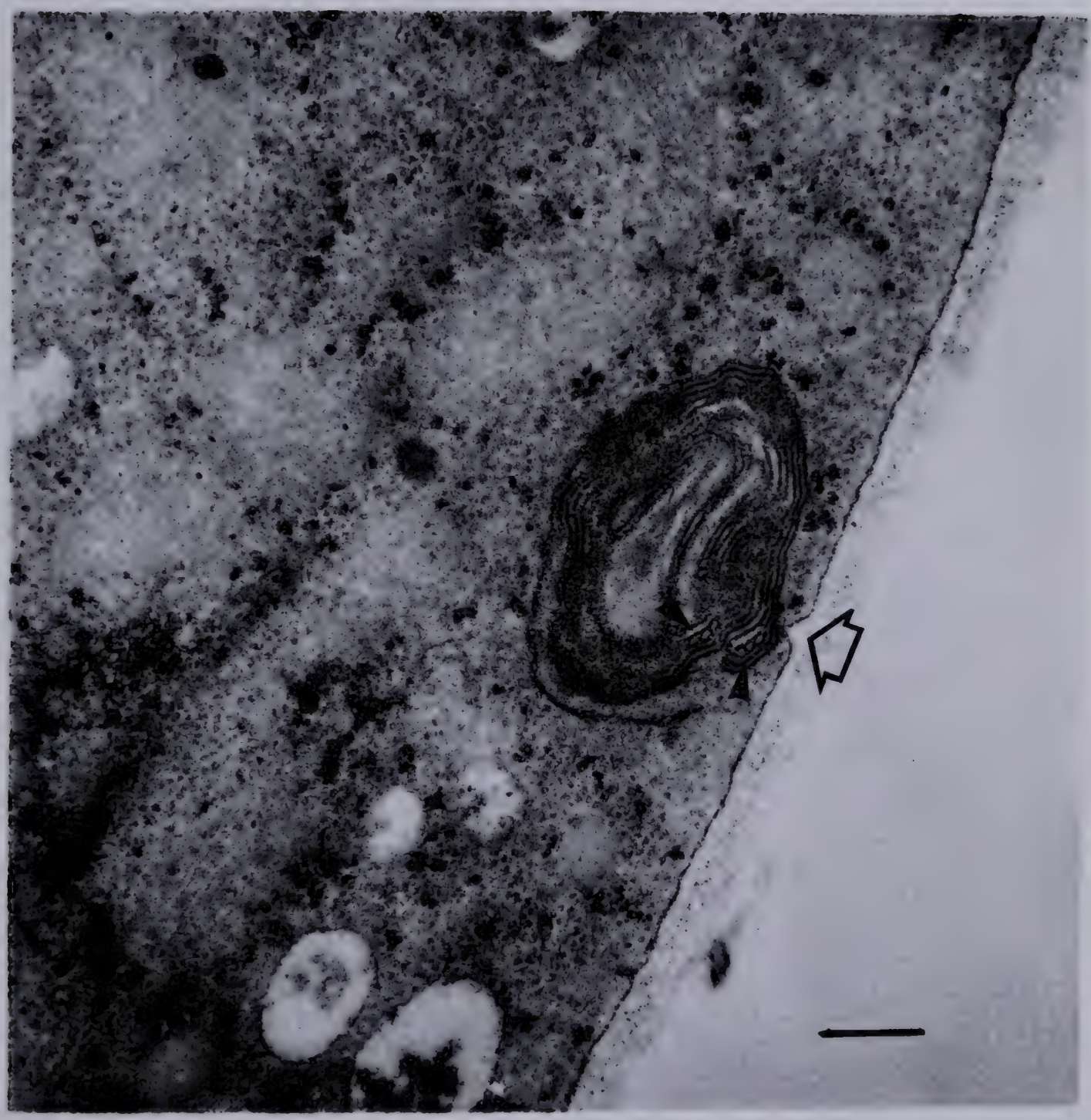


FIGURE 15

An electron micrograph of an exponentially growing Neurospora cell fixed only in glutaraldehyde and embedded in glycol methacrylate. A membrane can be seen connecting the mitochondrion with the plasma membrane (arrows). The plasma membrane is not discernible in this type of preparation as it appears white (electron transparent) against a white background. The section has been stained with hot uranyl acetate, and the calibration line represents 0.1 micron. The micrograph was taken with the specimen tilted 6^{o} clockwise from horizontal.

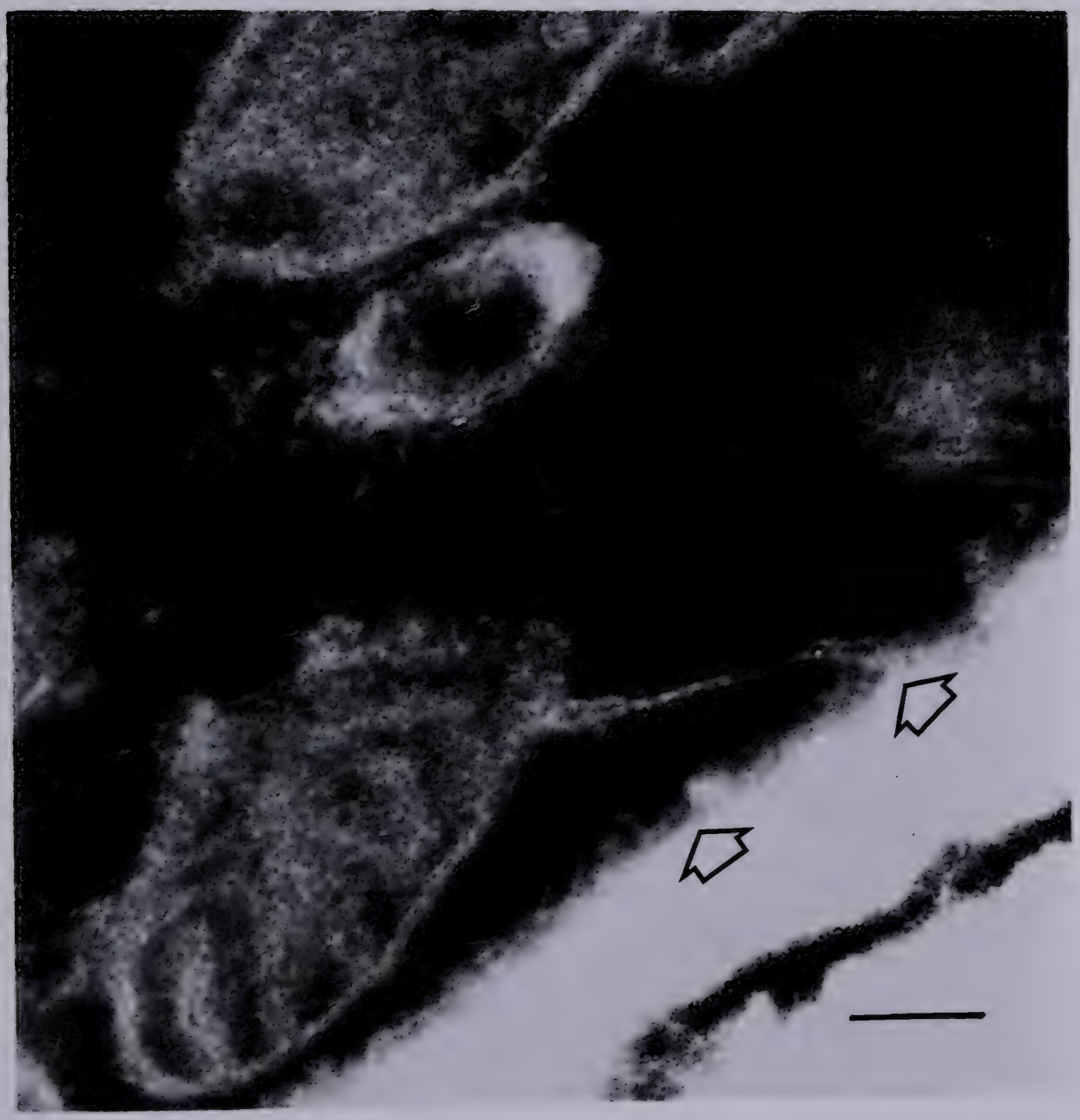
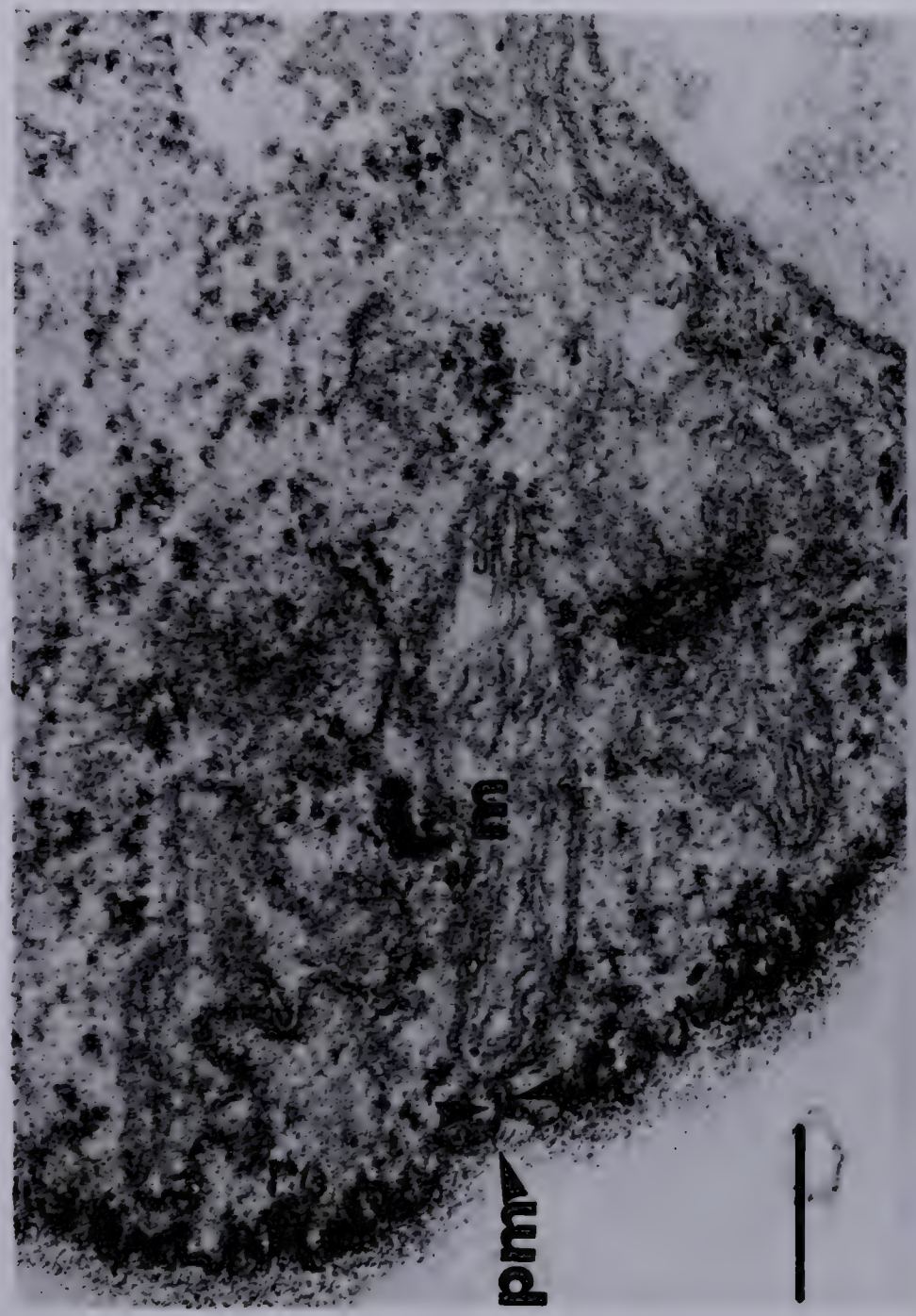


FIGURE 16

Two electron micrographs of the same cell of Neurospora from different angles. The material was fixed with OsO_4 only, from the early exponential growth phase and stained with lead citrate. An apparent connection can be seen (arrows) between the plasma membrane (pm) and the outer membrane of a mitochondrion (m). The upper micrograph was taken with the specimen tilted 27° clockwise from horizontal, and the lower 36° clockwise from horizontal. The calibration line represents 0.1 micron.


pm
m

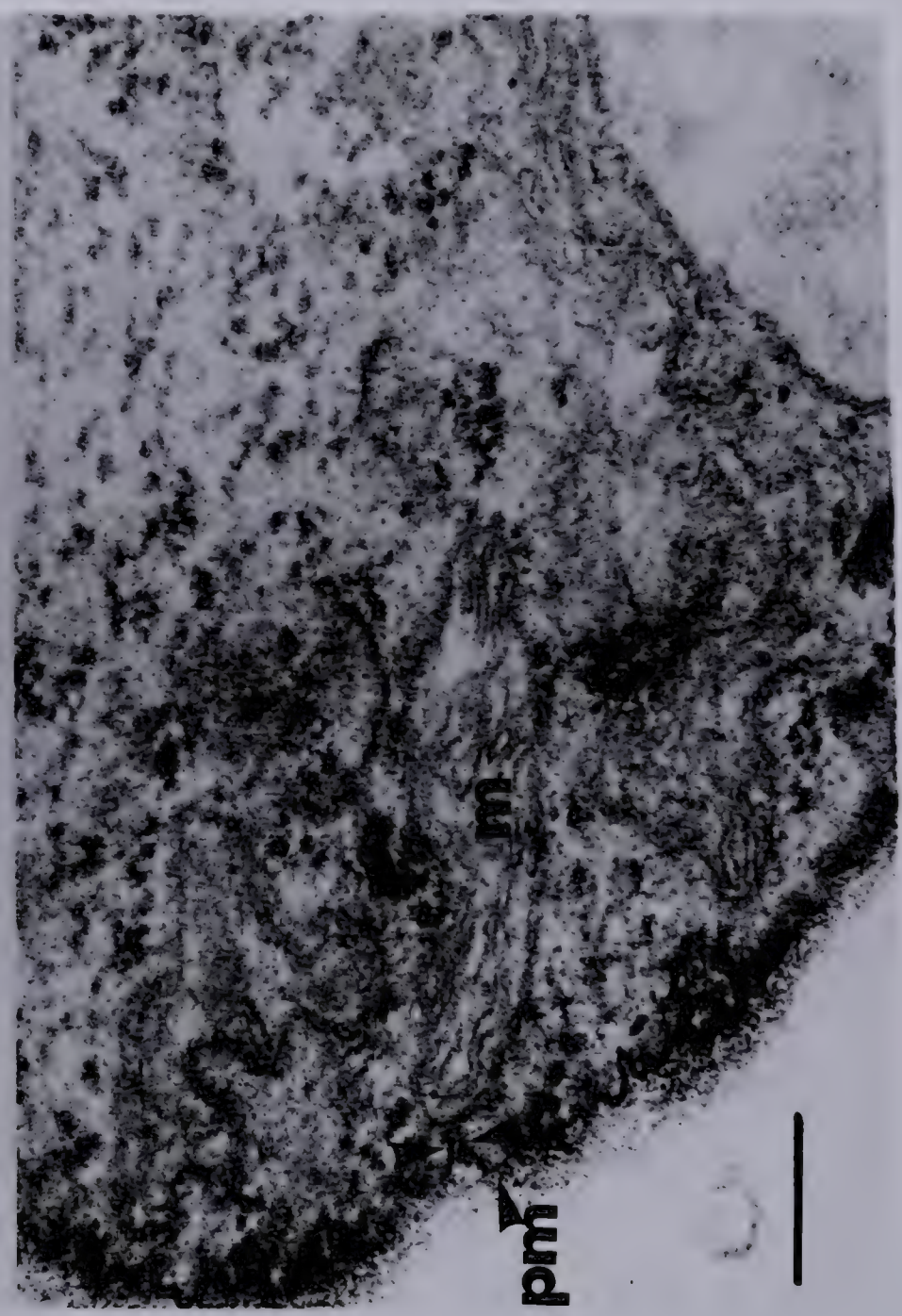

pm
m

FIGURE 17

An electron micrograph of a cell of Neurospora fixed only with OsO_4 from the early exponential growth phase. An apparent connection between the plasma membrane (arrows) and a mitochondrion (m) can be seen. This micrograph was taken with the specimen tilted 18^{o} counterclockwise from horizontal. The material is stained with lead citrate, and the calibration line represents 0.5 micron.

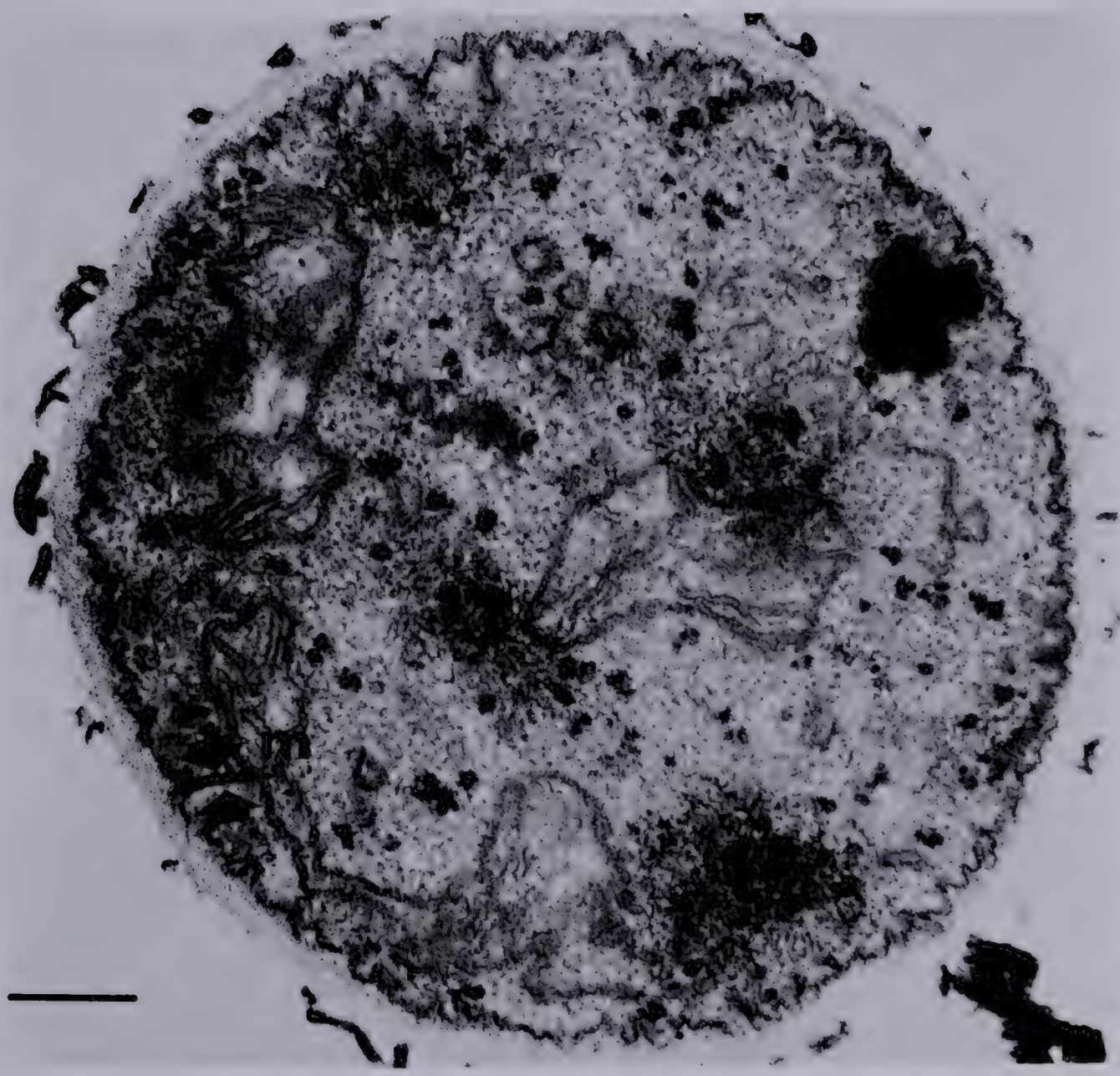


FIGURE 18

Table of plasma membrane length measurements from micrographs. The cells measured were from the same culture, were fixed at the same time, and were embedded by the same technique. The only difference in procedure was the method of fixation.

I. CELLS FIXED ONLY IN OsO_4

Cell wall length in measured segment	Plasma membrane length in same segment		Ratio PM length to CW length
687.2 mu	1231.2		1.82
921.2	1473.9		1.60
498.2	905.7		1.82
474.8	926.6		1.95
646.4	1189.8		1.84
		Average	1.81

II. CELLS FIXED IN GLUTARALDEHYDE AND OsO_4

692.3	749.9		1.08
652.1	669.2		1.03
794.1	915.3		1.15
823.8	909.4		1.10
1065.1	1143.1		1.07
		Average	1.09

II. Functional Studies

A. Respiratory Activity

Histochemical and biochemical studies of respiratory activity, using succinic dehydrogenase (SHD) as a marker, demonstrated the presence of this activity in non-mitochondrial membranes of Neurospora.

In fresh cells processed in complete ferricyanide medium for the demonstration of SDH activity, as previously described, deposits of electron dense copper ferrocyanide were observed on the mitochondrial membranes, mesosomes, and the plasma membrane in electron micrographs (Fig. 19). Not all mitochondria showed the same degree of electron dense deposit, some revealed a uniform heavy deposit, while others showed a patchy deposit, and still others showed no deposit at all. The plasma membrane and mesosomal membranes showed a similar heterogeneous response. Sections of fresh cells incubated in the ferricyanide medium without succinate showed sparse deposit on mitochondrial membranes, and no deposit on the plasma and mesosomal membranes. No electron dense deposits were seen in sections of fresh cells incubated in media to which malonate had been added. In sections of cells pre-fixed before incubation in the complete ferricyanide medium, a slight deposit was observed on the mesosomes or plasma membrane. No deposit at all was seen in sections of pre-fixed cells incubated in ferricyanide media from which

either succinate had been deleted or malonate added.

Incubation of fresh cells of Neurospora in TNBT medium gave results essentially similar to those obtained with the ferricyanide technique. Because of difficulty recognizing the histochemical reaction product in thin sections in the electron microscope, which apparently resulted from the low electron density of the formazan, the ferricyanide method was preferred. Experiments to determine the amount of electron density yielded by varying concentrations of formazans gave the results illustrated in Figure 20. This graph, plus subjective visual observations of the grids from which the optical (electron) density values were obtained, indicate that the amount of formazan necessary for consistant recognition is about 140×10^{-9} g/u^2. This value was determined by dividing the amount of formazan on the grid (1 mg) by the area of the grid (7.07 mm^2) which equals 0.14 mg/mm^2, or 140×10^{-9} g/u^2.

These histochemical results indicate an extramitochondrial SDH activity associated with the plasma and mesosomal membranes. Biochemical assays for SDH activity in fractions isolated from Neurospora cells showed SDH activity present in the microsomal fraction (Fig. 21), which should contain the fragmented plasma and mesosomal membranes. This SDH activity was found to be linear with time and protein concentration, and made up about 8 % of the total activity

(mitochondrial plus microsomal). No SDH activity was found associated with the soluble fraction of the cell. Examination of negatively stained samples of the microsomal fraction showed a small amount of mitochondrial contamination in this fraction. However, about 98 % of the F_1 ATPase activity was found in the mitochondrial fraction, and less than 2 % could be detected in the microsomal fraction (Fig. 21). These observations suggest that only about 25 % of the microsomal SDH activity can be accounted for by mitochondrial contamination. These data indicate that SDH activity is associated with the plasma and mesosomal membranes, as well as with the mitochondrial membranes, in Neurospora.

In parallel studies localizing SDH activity in rat liver cells, a similar situation was observed. In fresh cells processed histochemically by the ferricyanide technique for demonstration of SDH activity, electron dense deposits were observed on mitochondrial membranes, and on the plasma membranes of some cells. These cells also appeared structurally disorganized with considerable damage apparently occuring during the incubation procedure (Fig. 22). Cells fixed before incubation in the ferricyanide medium showed no SDH activity, but appeared morphologically intact. The electron dense deposit observed in cells treated by the ferricyanide technique apparently resulted from SDH activity,

as it was not observed if succinate was deleted or malonate was added to the incubation medium.

In subcellular fractions of rat liver cells, 92.1 % of the total SDH activity (mitochondrial plus microsomal) was recovered in the mitochondrial fraction and 7.9 % in the microsomal fraction. The microsomal fraction was further sub-fractionated into rough and smooth fractions, and 73.7 % of the microsomal activity was recovered in the smooth fraction. Twenty-two per cent of the microsomal activity was found in the rough fraction. The mitochondrial fraction contained 95 % of the F_1 ATPase activity, and 5 % of this activity was found in the microsomal fraction, indicating that about 70 % of the microsomal SDH activity could be accounted for by mitochondrial contamination.

Despite this large amount of apparent mitochondrial contamination of microsomal material, the histochemical results together with the microsomal SDH activity which cannot be accounted for by contamination, strongly suggest the presence of extra-mitochondrial SDH activity in these cells. These results also suggest that the extra-mitochondrial presence of SDH activity may be a more general feature of cells, as it has been demonstrated here in two widely different cell types.

FIGURE 19

Three electron micrographs of Neurospora showing the dense reaction product resulting from the ferricyanide technique for demonstration of SDH activity in (A) a mitochondrion, (B) a mesosome, and (C) the plasma membrane. Arrows in B and C indicate reaction product. Magnification of A is 82,800, B is 101,200, and C is 205,100. The material is unstained.

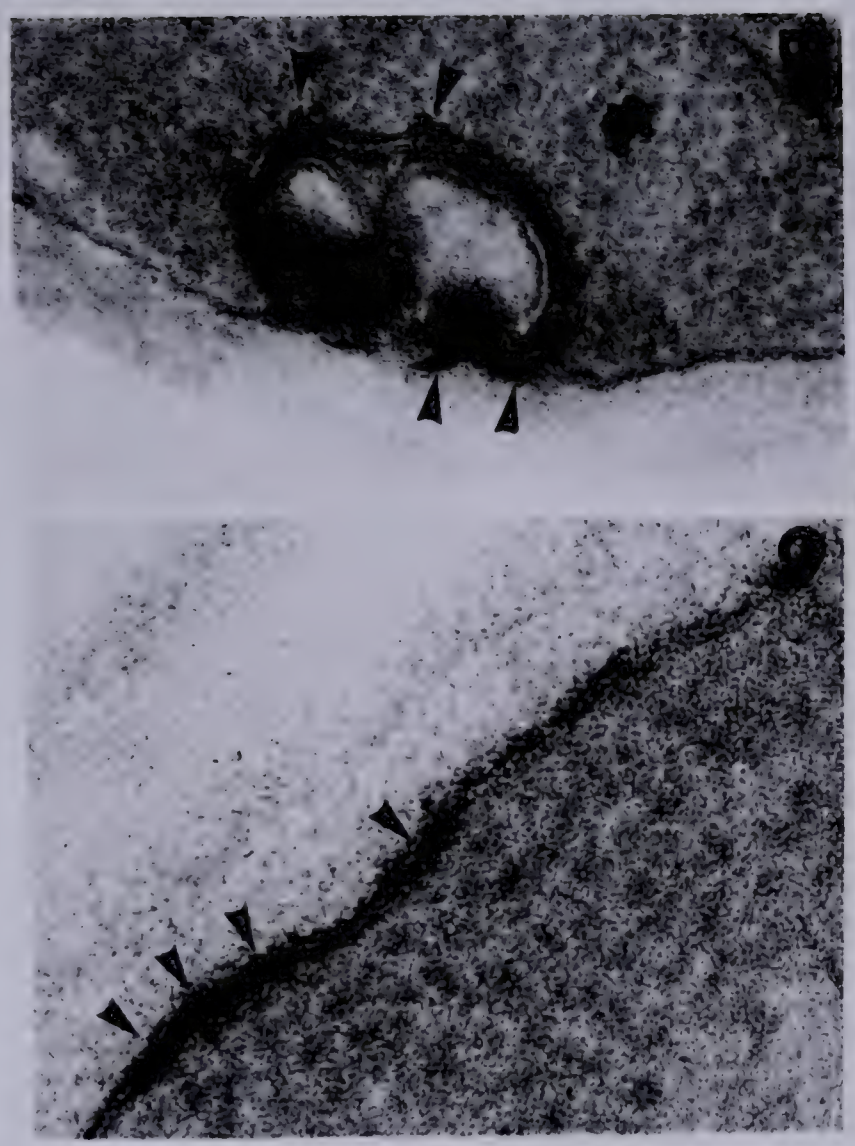


FIGURE 20

A graph illustrating the relation of optical density to various concentrations of formazan on grids photographed in the electron microscope. Each point represents an average of 20 values taken from different points on each grid. Two grids of each concentration were photographed. ΔO. D. represents the difference in optical density between the naked formvar film and the formvar film coated with formazan.

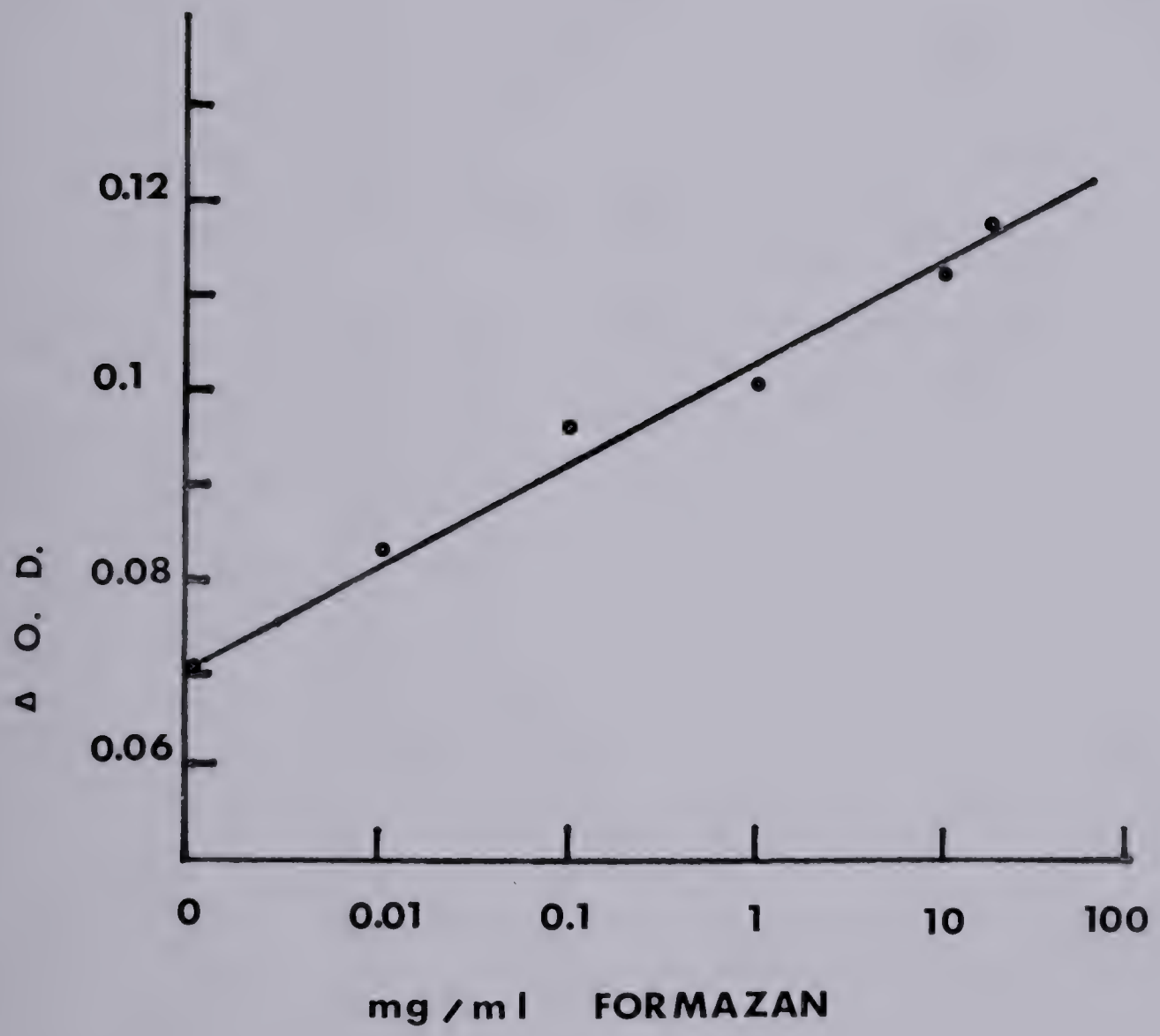

0.12
0.1
0.08
0.06
Δ O. D.
0
0.01
0.1
1
10
100
mg / ml FORMAZAN

FIGURE 21

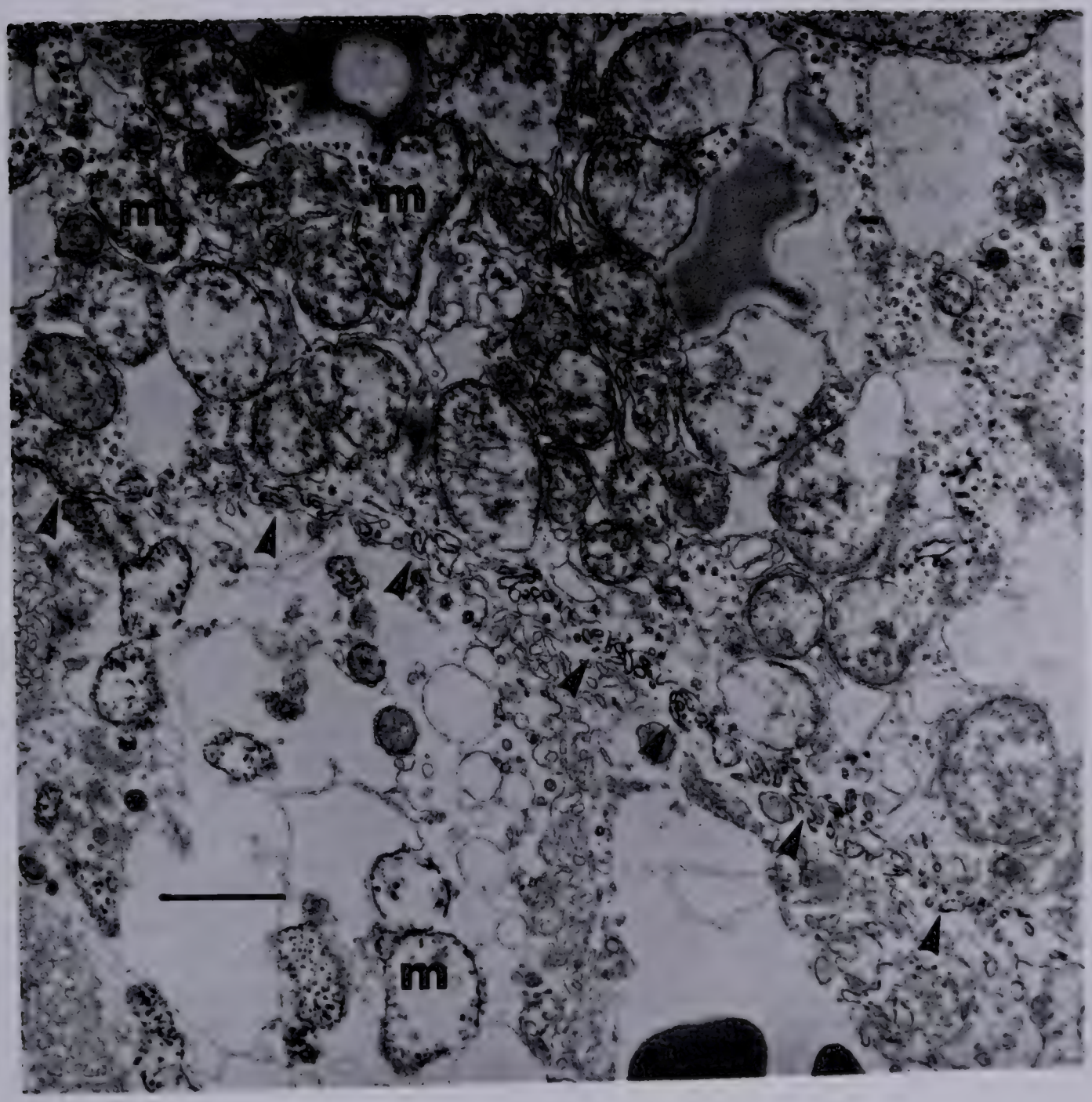
Summary of SDH and F_1 ATPase activities in fractions of Neurospora. SDH units equal the reduction in optical density at 600 mu per minute per mg protein at 37°C, and F_1 ATPase units equal ug Pi released per minute per mg protein at 37°C.

SUMMARY OF SDH, F_1 ATPase ACTIVITY IN FRACTIONS OF NEUROSPORA

Exp.	Mitochondrial SDH Activity	Microsomal SDH Activity	% Total SDH Activity As Microsomal	Mitochondrial F_1ATPase Activity	Microsomal F_1ATPase Activity	% Total F_1ATPase as Microsomal
1	0.21	0.010	9.40	-	-	-
2	0.20	0.030	9.60	-	-	-
3	0.17	0.020	8.40	-	-	-
4	0.24	0.016	6.25	-	-	-
5	0.25	0.018	6.70	5.43	0.21	2.3
6	-	-	-	6.81	0.09	0.9
7	0.24	0.030	7.50	5.80	0.10	1.1
Averages			7.97			1.4

FIGURE 22

An electron micrograph of rat liver tissue treated for demonstration of SDH activity. Deposits of electron dense material can be seen along the plasma membrane of one of the cells (arrows), as well as in the mitochondria (m). The material was doubly fixed after treatment for SDH demonstration, and is unstained. The calibration line represents 1.0 micron.


m
m
m

Mitochondrial DNA Replication

Investigations of factors affecting incorporation of ^{3}H-TTP by isolated mitochondrial fractions from Neurospora demonstrated that this incorporation does take place, and is influenced by: (1) the isolation procedure; (2) the time lapse between isolation of the mitochondria and assaying of ability to incorporate ^{3}H-TTP; (3) the composition, temperature, and pH of the assay medium. Also affecting this incorporation are two enzymatic reactions which apparently occur concomitantly with incorporation of ^{3}H-TTP. One is a dephosphorylation, converting ^{3}H-TTP to ^{3}H-TMP, and the second is a nuclease activity which could degrade both the newly formed and pre-existing nucleic acid in the mitochondria.

Isolated mitochondria incorporated 2.8×10^{-12} moles of ^{3}H-TTP per mg of mitochondrial protein into acid insoluble material in 15 minutes under optimal conditions. This figure is an average of 9 determinations. This activity varied linearly with protein concentration in the range tested (0.25 mg/ml to 2.0 mg/ml). The extent and rate of the incorporating activity was influenced by the method of purification of the mitochondria. Mitochondria prepared by differential centrifugation without subsequent purification on density gradients were found to be more active (3.7 versus 2.8×10^{-12} moles/mg protein/15 minutes) than mitochondria purified by density gradient centrifugation. Electron

microscopic examination of negatively stained samples showed mitochondria isolated by differential centrifugation alone were mostly intact, though somewhat swollen. However, these preparations were also contaminated by non-mitochondrial membranous material which proved difficult to remove without losing a considerable amount of mitochondrial protein. A single pass on the previously described density gradients removed almost all of this material, but some of the mitochondria were disrupted. This was observed by studying several negatively stained preparations obtained by density gradient centrifugation (Figs. 23, 24). Approximately 25 % of the mitochondria in these preparations appeared broken.

Maximum incorporation of ^{3}H-TTP was observed in mitochondrial preparations assayed soon after isolation. After 24 hours of storage, incorporating activity was reduced about 50 %, and after 48 hours, none could be detected.

The effect of composition, pH, and temperature of the assay medium on incorporation of ^{3}H-TTP is given in Figure 25. Each value is the average of three or more determinations. These results show that Mg^{++} and all four deoxynucleoside triphosphates are required for incorporation, that the pH optimum is 7.5, and that the temperature optimum is 27^{o}C. The temperature of the assay medium was observed to affect not only the rate of the reaction, but also the

total amount of ^{3}H-TTP incorporated (Fig. 26). At 27°C, the level of maximum incorporation was reached in about 15 minutes, whereas at 37°C a lower maximum was reached in about 5 minutes. At both these temperatures, a rapid decline in acid precipitable radioactivity occurred after peak incorporation was reached.

This rapid decline in acid precipitable radioactivity suggested the presence of a nuclease activity associated with the mitochondrial fraction. This fraction was therefore assayed for nuclease activity as previously described, and found to be highly active. The characteristics of the nuclease activity were very similar to those described by Linn and Lehman (32) for an endonuclease of Neurospora mitochondria. The nuclease was found to be effective on native or denatured DNA to the same extent. When the mitochondria were disrupted by sonication, the activity was found to increase by 60 % over the initial value. Storage of the mitochondria at 4°C for 3 days resulted in more than a 100 % increase over the initial value (Fig. 27). Addition of EDTA to the assay system abolished the nuclease activity of the mitochondrial fraction, and this effect of EDTA could be reversed by Mg^{++}. The nuclease activity was negligible when Ca^{++} was substituted for Mg^{++} (Fig. 28).

The activity of a nuclease degrading newly synthesized DNA labelled by incorporation of ^{3}H-TTP should result in the

release of ^{3}H-TMP as one of the degradation products. This was assayed for as described and found. However, the rate of appearance, and the amount of ^{3}H-TMP recovered, was much greater than expected (32). Even more ^{3}H-TMP was recovered if incorporation of ^{3}H-TTP was prevented by ommission of one or more of the unlabelled deoxynucleoside triphosphates from the assay medium (Fig. 29). This conversion was linear with time, and up to 50 % of the ^{3}H-TTP was converted to ^{3}H-TMP in 90 minutes. No conversion occurred without the presence of mitochondria during this time.

Isolated mitochondrial fractions from Neurospora treated by the detergent fractionation technique of Tremblay et al. (10), modified as described previously, were found to form membrane-detergent crystal complexes. If the DNA of these mitochondria was labelled by use of either ^{3}H-TTP or ^{3}H-actD before treatment with the detergent, some of the radioactivity was recoverable in this complex. When mitochondria with labelled DNA were centrifuged on control gradients (without detergent), 95.8 % of the acid insoluble radioactivity was recovered in the mitchondrial band (Fig. 30). When the detergent was added to these preparations, only 27 % of the original acid insoluble radioactivity was recovered, all of it in the membrane-detergent crystal complex (Fig. 30). If Mg^{++} was substituted for Ca^{++} in the gradient solutions, only about 1 % of the original acid in-

soluble radioactivity was recovered. Analysis of the total radioactivity of the fractions from the detergent containing gradients showed a high amount of radioactivity at the top of the gradients, as compared to the control gradients (Figs. 31, 32).

FIGURE 23

A low magnification micrograph of negatively stained mitochondria from Neurospora isolated by density gradient centrifugation. The mitochondria were stained with 2 % potassium phosphotungstate. The calibration line represents 1.0 micron.

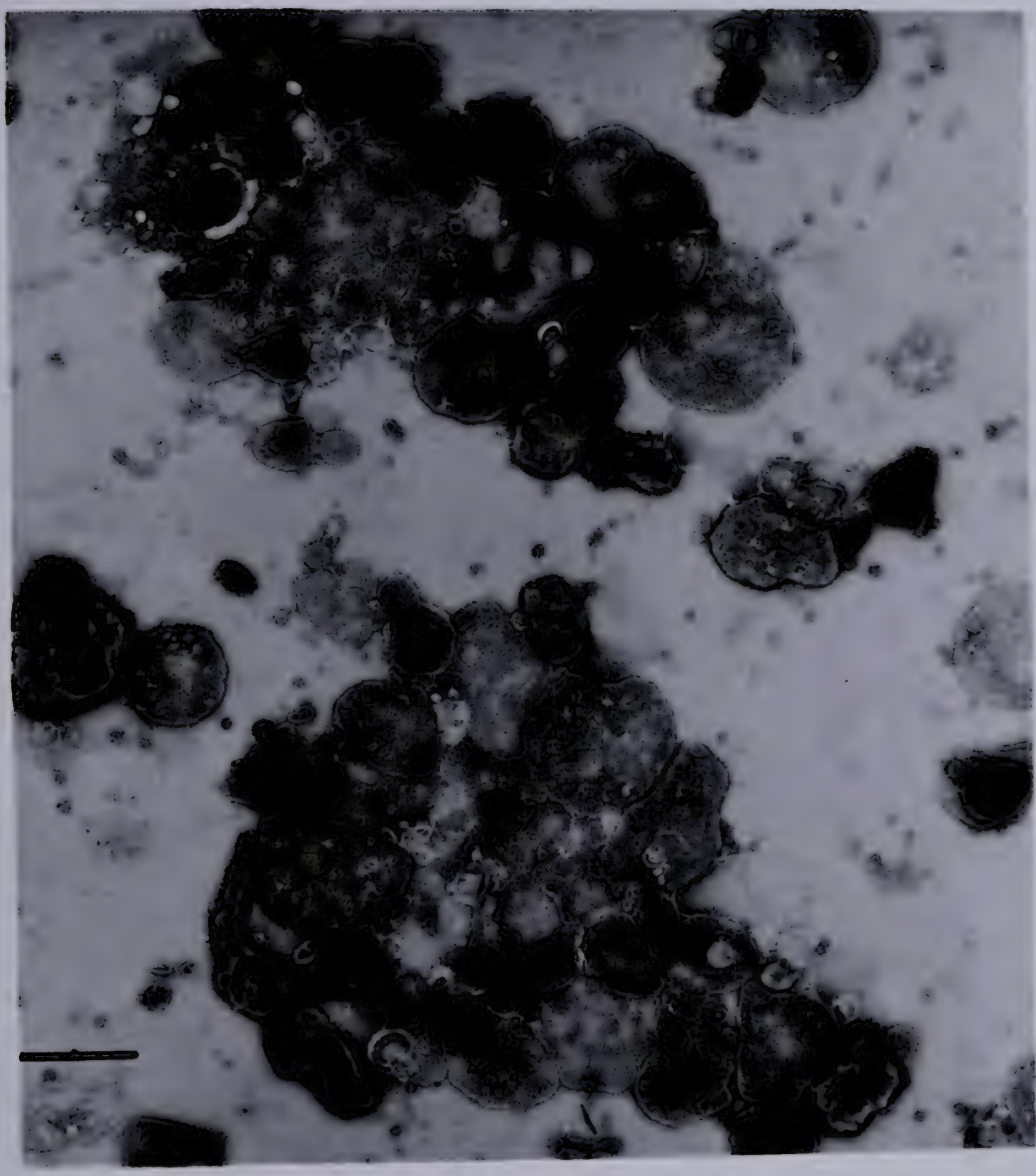
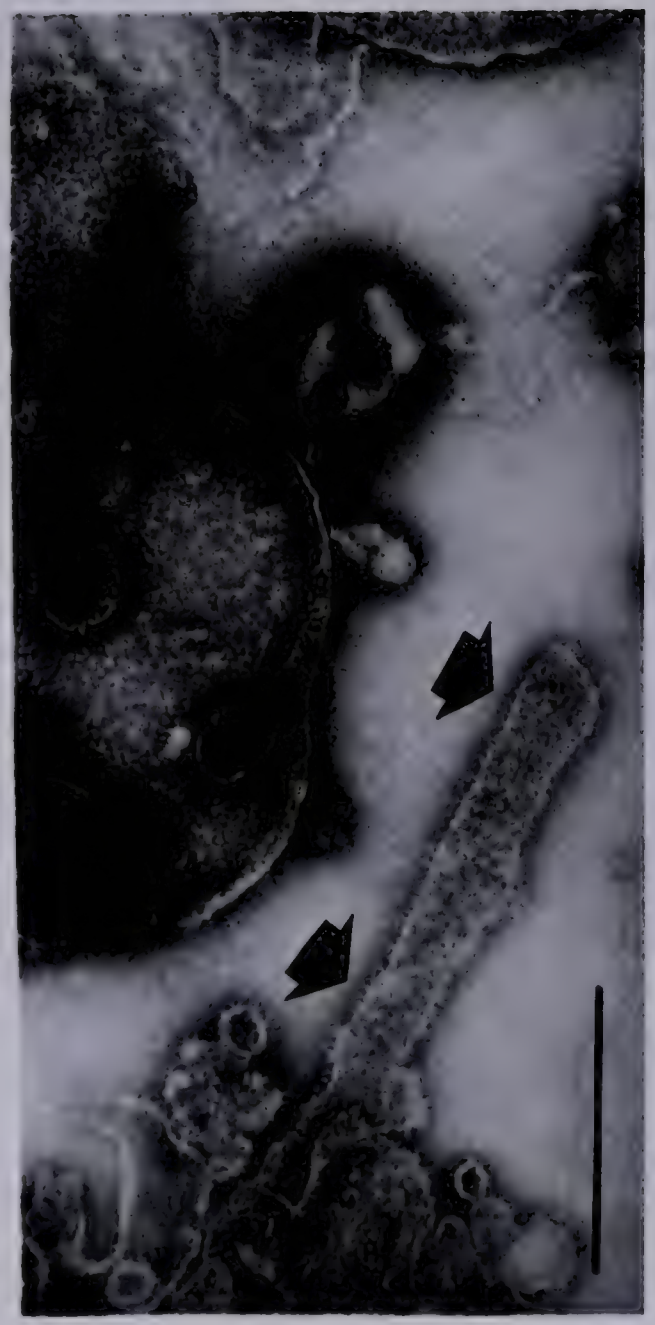


FIGURE 24

Electron micrographs of negatively stained mitochondria from Neurospora isolated by density gradient centrifugation. Both intact and disrupted mitochondria can be seen. At top right in the upper micrograph (arrow), elementary particles can be seen on disrupted mitochondrial membranes. The lower micrograph is an enlarged view of this area. The calibration line in the upper micrograph represents 1.0 micron, and in the lower, 0.5 micron.



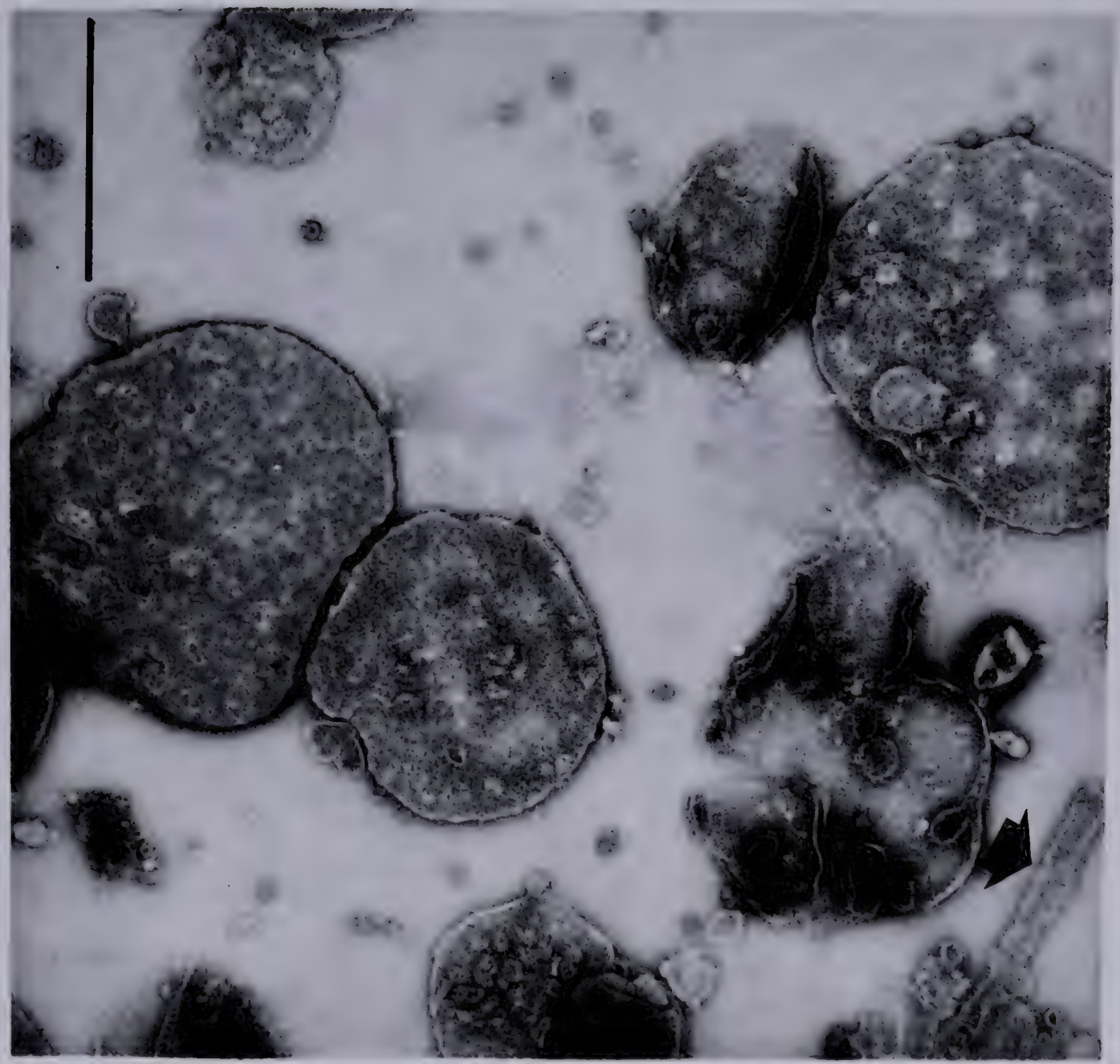


FIGURE 25

Table summarizing the effect of composition (top), pH (middle), and temperature (bottom) of the incorporation of ^{3}H-TTP by isolated mitochondria of Neurospora. The composition of the basic medium is given in the text.

Effect of composition, pH, and temperature on incorporation of ^{3}H-TTP by mitochondria of Neurospora.

Assay medium	moles x 10^{-12} ^{3}H-TTP incorporated in 15 minutes per milligram mitochondrial protein
Basic, at 27°C, pH 7.5	2.8
Basic, - dATP	0.1
Basic, - dCTP	0.7
Basic, - dGTP	0.7
Basic, - dCTP, dGTP	0.0
Basic, + ATP (1.0 umole/ml)	2.2
Basic, + ATP, - dATP	1.0
Basic, - Mg^{++}	0.0
Basic, - ^{3}H-TTP, + ^{3}H-thymidine	0.0
Basic, pH 6.5	0.6
Basic, pH 7.0	1.1
Basic, pH 7.5	2.8
Basic, pH 8.0	0.7
Basic, 0°C	0.0
Basic, 27°C	2.8
Basic, 37°C (in 5 minutes)	1.7
Basic, 45°C (in 5 minutes)	1.1

FIGURE 26

Graph illustrating the effect of temperature on the incorporation of ^{3}H-TTP by isolated mitochondria of Neurospora at 37°C (circles), and 27°C (x's). Each point is an average of three or more determinations.

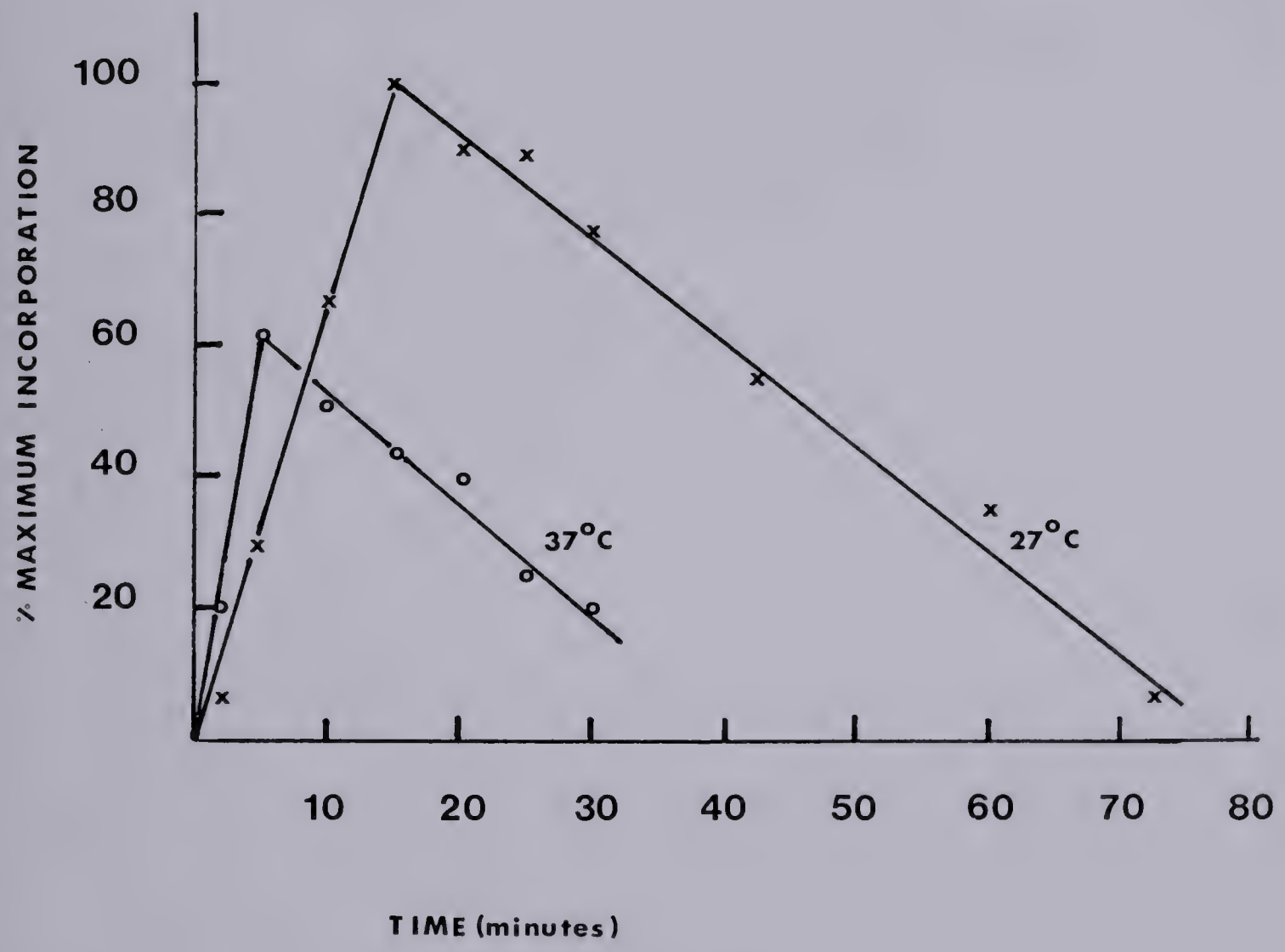

% MAXIMUM INCORPORATION
100
80
60
40
20
37°C
27°C
10
20
30
40
50
60
70
80
TIME (minutes)

FIGURE 27

The effect of sonication (top) and storage (bottom) on the nuclease activity associated with mitochondria of Neurospora.


200
180
160
140
120
100
1
2
3
4
5
MINUTES SONICATED
PER CENT INITIAL ACTIVITY

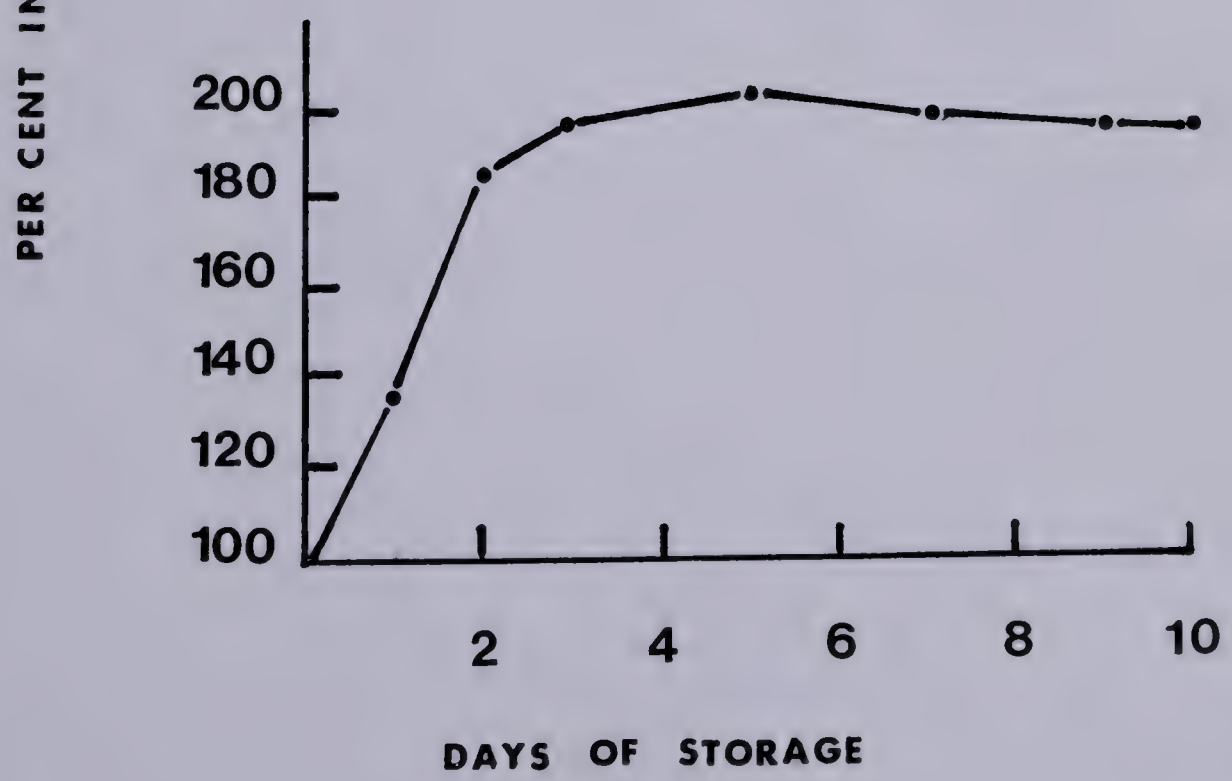

200
180
160
140
120
100
2
4
6
8
10
DAYS OF STORAGE

FIGURE 28

Nuclease activity of mitochondria of Neurospora. Each assay contained 1.0 ug mitochondrial protein. $CaCl_2$ was present at 1.0 umole per assay when used in place of $MgCl_2$.

Endonuclease activity associated with mitochondria of Neurospora

Preparation	Enzyme units
Basic assay* with native DNA	2.9
Basic assay with denatured DNA	3.5
Basic assay - $MgCl_2$, + $CaCl_2$, native DNA	0.3
Basic assay + 0.005 M EDTA, no cation, native DNA	0.0
Basic assay + 0.005 M EDTA, 0.01 M $MgCl_2$, native DNA	2.4

* As described in Materials and Methods

FIGURE 29

Conversion of ^{3}H-TTP to ^{3}H-TMP by mitochondria of Neurospora. The upper line (solid circles) represents the percent of acid soluble dpm as TMP versus time in the absence of ^{3}H-TTP incorporation by mitochondria. The lower line (open circles) represents the percent of acid soluble dpm as TMP in the presence of ^{3}H-TTP incorporation by mitochondria.

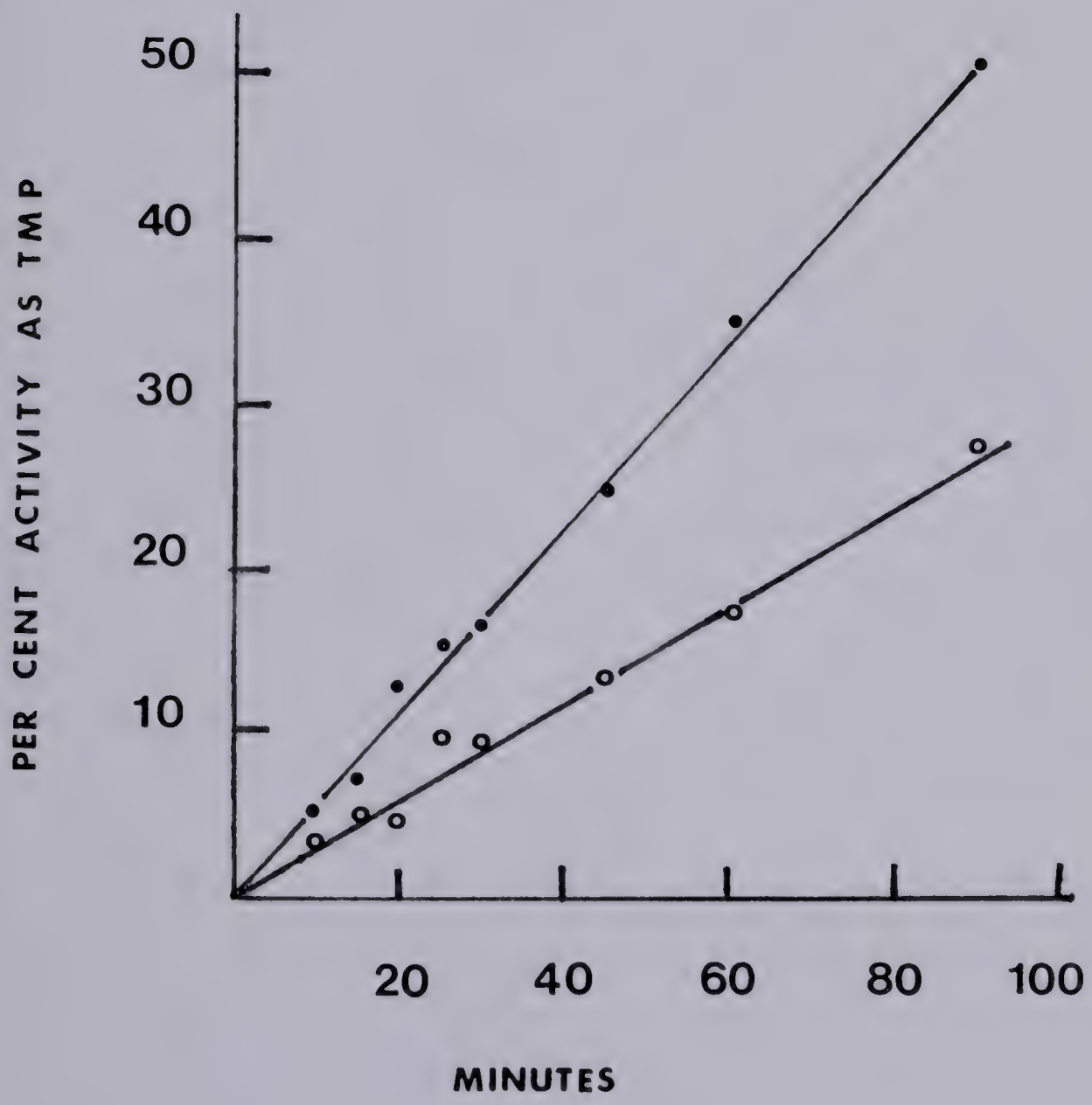

PER CENT ACTIVITY AS TMP
50
40
30
20
10
20
40
60
80
100
MINUTES

FIGURE 30

Density gradient fractionation results from untreated (upper graph) and detergent treated (lower graph) mitochondria labelled with ^{3}H-TTP. The solid line represents the optical density at 280 mu of the fractions, and the dotted line represents the acid insoluble radioactivity (dpm) of the fractions.

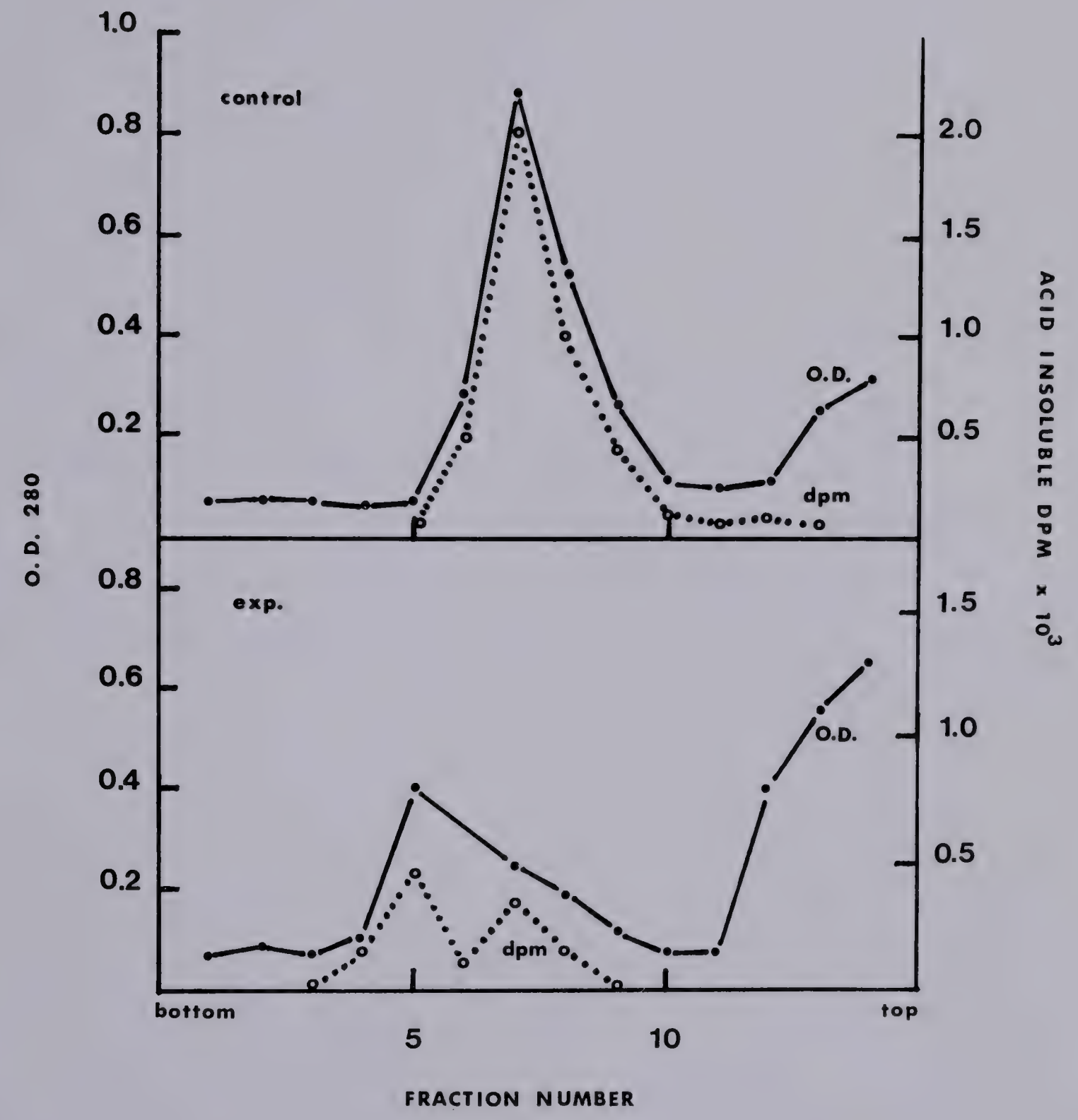

control
exp.
O.D.
dpm
O.D.
dpm
1.0
0.8
0.6
0.4
0.2
0.8
0.6
0.4
0.2
2.0
1.5
1.0
0.5
1.5
1.0
0.5
O. D. 280
ACID INSOLUBLE DPM x 10^3
bottom
top
5
10
FRACTION NUMBER

FIGURE 31

Density gradient fractionation results from untreated (upper graph) and detergent treated (lower graph) mitochondria labelled with ^{3}H-TTP. The solid line represents the optical density at 280 mu of the fractions, and the dotted line represents the total radioactivity (dpm) of the fractions.

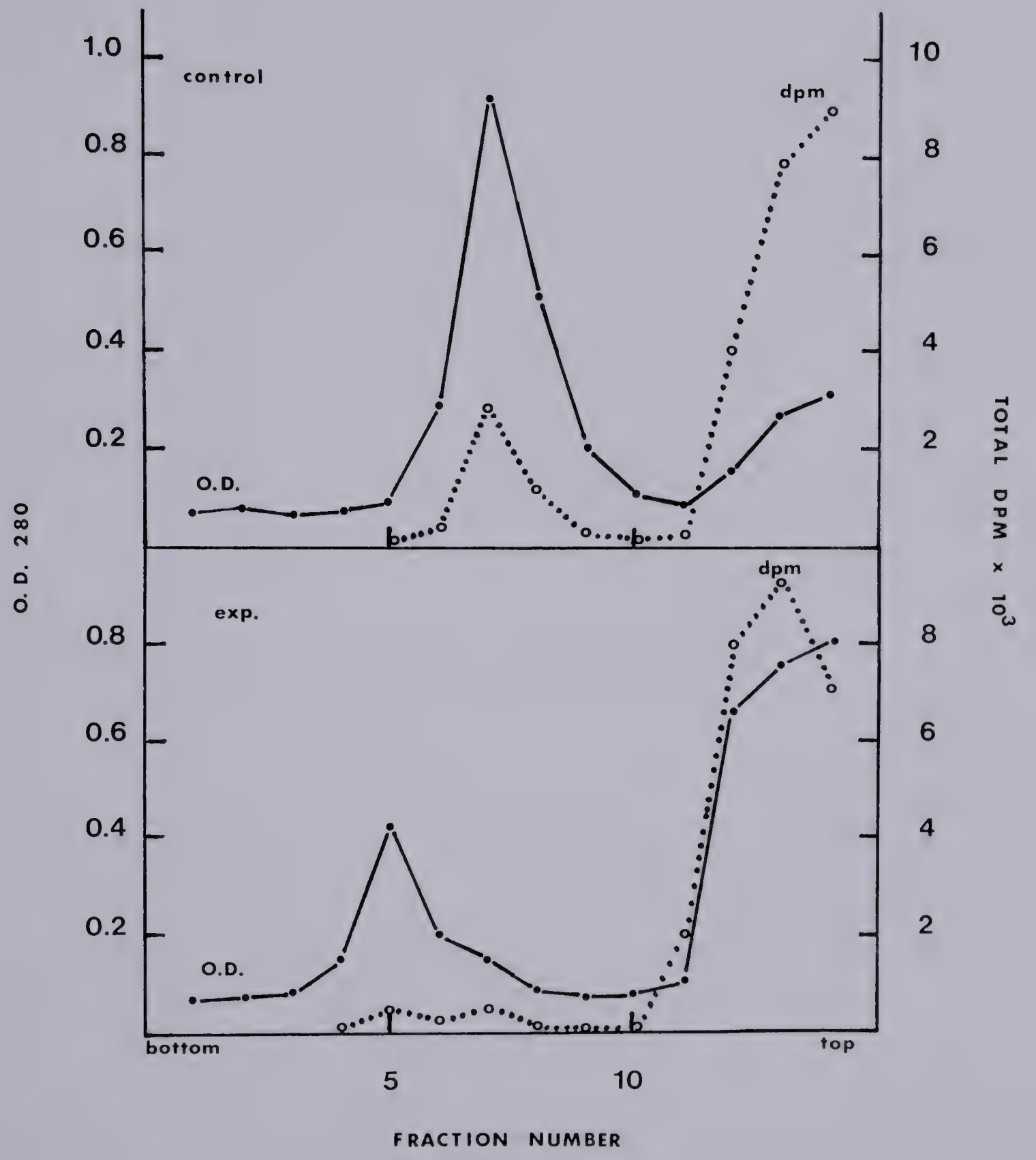
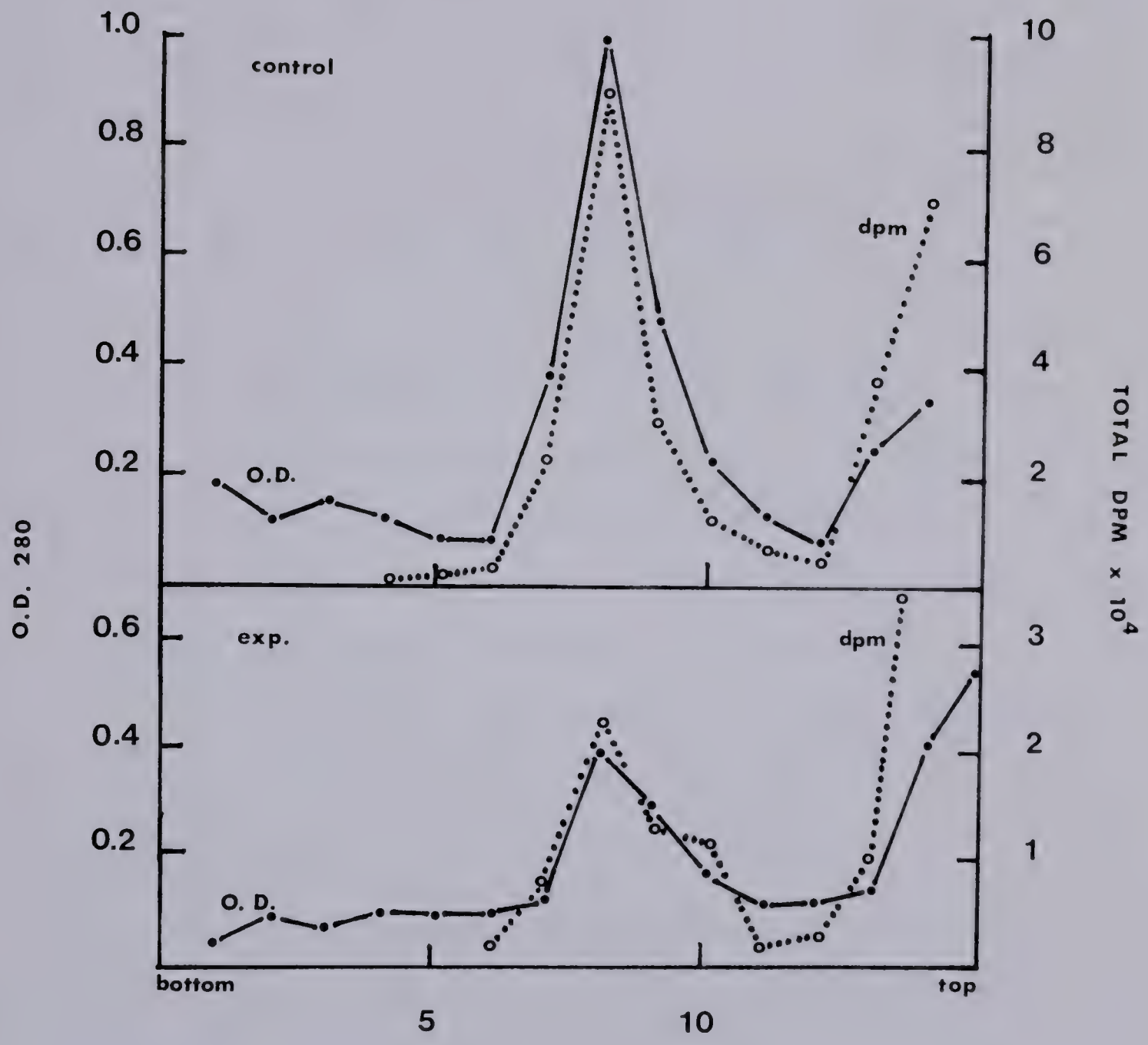

control
dpm
O.D.
1.0
0.8
0.6
0.4
0.2
10
8
6
4
2
exp.
dpm
O.D.
bottom
top
5
10
FRACTION NUMBER
O. D. 280
TOTAL DPM x 10³

FIGURE 32

Density gradient fractionation results from untreated (upper graph) and detergent treated (lower graph) mitochondria labelled with ^{3}H-actinomycin D. The solid line represents the optical density at 280 mu of the fractions, and the dotted line represents the total radioactivity of the fractions.


control
O.D.
dpm
exp.
O. D.
dpm
1.0
0.8
0.6
0.4
0.2
0.6
0.4
0.2
10
8
6
4
2
3
2
1
O.D. 280
TOTAL DPM x 10^4
bottom
top
5
10
FRACTION NUMBER

DISCUSSION

I. Morphological Studies

The results of the morphological studies on mesosomes of Neurospora suggest several things. One is that the morphology seen in a section at a given time of culturing is not a direct function of the duration of culturing, or the growth stage, but where in the cell the section was made. The frequency of observation of mesosomes can be seen to be correlated to particular growth stages during culturing (Fig. 7). This could be interpreted to indicate that mesosomes are characteristics of this particular growth stage. However, because sectioning of a sample of Neurospora hyphae is a random selection of areas (sections) of cells for observation, this interpretation implies that the hyphae observed are representative of the entire culture, and that the hyphae are morphologically homogeneous along their length.

A variety of observations suggest that this is not true. It has been shown that physiological differentiation in hyphae of Neurospora does exist, and that growth occurs only at the tip of the cell (33-35). Examination of serial sections of individual cells is difficult because of the long length of the cell compared to the thickness of the

sections (the growing region alone will yield over 300 sections of 600Å thickness). However, examination of fortuitously obtained longitudinal sections do show general morphological differences along the length of the cell (Fig. 33). It therefore seems likely that the apparent association of mesosomes with the early growth stages is probably a result of mesosomes being a characteristic of the growing zone of the cell. At the stage when conidia are germinating, growing zones are much more abundant than at other stages, and make up most of the length of the cell. If mesosomes are a characteristic of the growing zone, then they will be observed most often in material fixed at this stage. At later stages, the proportion of the growing zone to the rest of the cell becomes less and less, and so does the frequency of observation of mesosomes.

The effect of different fixatives on the ultrastructural appearance of cells of Neurospora is marked. There is an overall difference in the appearance of membranous structures in doubly fixed cells (glutaraldehyde followed by OsO_4) as compared to cells fixed only in OsO_4. In doubly fixed cells, membranes have a relatively smooth appearance, stain intensely (except the mitochondrial membranes), and appear well defined. In cells fixed only with OsO_4, all membranes appear highly irregular and convoluted (Fig. 34), stain poorly, and do

not appear well defined. Complex mesosomes are not seen in cells fixed only with OsO_4. An association between simple infoldings of the plasma membrane and mitochondrial membranes is often seen in cells fixed with either glutaraldehyde or OsO_4, but not both. In some of these preparations the plasma membrane appears to be continuous with the outer mitochondrial membrane (Figs. 15, 16, 17).

Curgy (5) has observed structures similar to mesosomes of Neurospora in embryonic chick hepatocytes after double fixation. He does not observe them after fixation in OsO_4 alone. For this reason he suggests that these mesosome-like structures are reorganization products of membrane somehow mobilized or disorganized by glutaraldehyde (which is not a lipid fixative) and then fixed in a new organization by OsO_4. Similar structures to Curgy's mesosomes can be produced in mouse heart mitochondria by allowing the heart tissue to sit at room temperature for 30 minutes in buffer after removal from the mouse and before fixation (Fig. 35). These structures are seen with double fixation after this treatment, but not if the heart tissue is fixed immediately upon removal from the animal.

Gupta and Malhotra (36) have performed experiments designed to demonstrate penetration of lanthanum hydroxide into mesosomes of Neurospora from the outside medium, using the technique of Revel and Karnovsky (37). These studies

were performed to elucidate the physical relationship between the plasma membrane, mesosomal membranes, and mitochondria. Lanthanum was used for this purpose because of its observed ability to penetrate intracellular spaces without penetrating cells, and because of its high electron density. In cells prefixed with glutaraldehyde and incubated with lanthanum in the presence of OsO_4, lanthanum was observed in the interior of mesosomes, including those associated with mitochondria. Attempts to show lanthanum penetration in unfixed cells, or cells fixed only with glutaraldehyde were unsuccessful. The authors suggest that OsO_4 fixation is necessary to retain lanthanum in tissues during subsequent processing for electron microscopy, as lanthanum deposits were not seen when the tissue was fixed with aldehyde alone. However, it seems equally possible that double fixation may be necessary for lanthanum penetration into mesosomes, either by physically altering the membrane, or by causing a reorganization of membrane material as suggested by Curgy (5). It is known that OsO_4 reacts with lipids of membranes by cross linking unsaturated fatty acids through diester formation, causing polymerzation of the fatty acids (38). Because lanthanum penetration cannot be demonstrated without OsO_4 fixation, it seems likely that some alteration to the membrane is necessary for this penetration to be observed.

Observations of fresh frozen-etched cells of Neurospora

by Tewari (39) have shown no structures comparable to mesosomes as seen in sections from doubly fixed cells. These same preparations show smooth membrane surfaces similar to the profiles seen in sections of doubly fixed cells, and not convoluted as seen in cells fixed only in OsO_4 (Fig. 36). However, mesosome-like structures can be seen in frozen-etched preparations of Neurospora if the cells were pre-fixed briefly with glutaraldehyde before freezing.

The bulk of this evidence suggests that mesosomes, as seen in double fixed cells, could be reorganized configurations of some cellular membranes. The fact that enzymatic activity (succinic dehydrogenase) can be demonstrated in association with mesosomal membranes and the plasma membrane, and that doubly fixed cells show 40 % less plasma membrane length in electron micrographs, suggests mesosomes of Neurospora could be reorganization products derived from the plasma membrane during fixation. The connections observed between the plasma membrane and mitochondria could provide some of the material and a site for formation of mesosomes, and might explain the close association of some mesosomes with mitochondria. Figure 37 shows apparent reorganization of unit membrane structure within a large complex mesosome to a system of tubules reminiscent of micellar arrays.

Two considerations argue against this conclusion. One

is the observations of freeze-etch preparations of Neurospora which show membrane surfaces which are not convoluted as seen in cells fixed only in OsO_4, but are smooth and similar to the profiles seen in doubly fixed cells. Also, no communicating membranes between the plasma membrane and mitochondria have been observed in freeze-etch preparations of Neurospora (39). Second, mesosomes seen in doubly fixed cells have never been observed to be continuous with the mitochondrial membranes, as would be expected if they were derived from communicating membranes between the plasma membrane and mitochondrial membranes.

For these reasons, it is difficult to come to a conclusion about the reality of mesosomes in Neurospora. Because they can be seen routinely in doubly fixed cells, and because enzymatic activity can be demonstrated associated with them, it seems likely that while they may not exist in vivo in the form in which they are observed in doubly fixed material, they are a result of some real "structure" which does exist in vivo and is somehow modified in different ways by different fixation procedures. This "structure" probably is a modification of the plasma membrane. More evidence for this is provided by the functional studies discussed below.

FIGURE 33

A long section of a germinating conidium. This figure is a montage of 3 prints, and has been reduced considerably in size to fit on the page. The conidium is the expanded portion of the cell to the bottom left. The growing region of the cell is approximately the upper half of the hypha. No septa have formed. The cytoplasm of the tip region appears densely packed with glycogen granules and ribosomes. Numerous small mitochondria are present in this region, and nuclei and vacuoles are not seen. Also, very few lipid droplets are present in the tip region. In the half of the hypha adjoining the conidium, nuclei are present, mitochondria are larger and more complex, and lipid droplets are much more abundant. The cytoplasm does not appear as densely packed with glycogen and ribosomes. This cell was fixed with glutaraldehyde and OsO_4. The calibration line represents 1.0 micron.

FIGURE 34

An electron micrograph of a Neurospora cell fixed in OsO_4 only, from the exponential phase of growth, and stained with lead citrate. The plasma membrane (pm), and nuclear membranes (N) all appear irregular and convoluted as compared to similar structures seen in doubly fixed cells. The calibration line represents 0.1 micron.

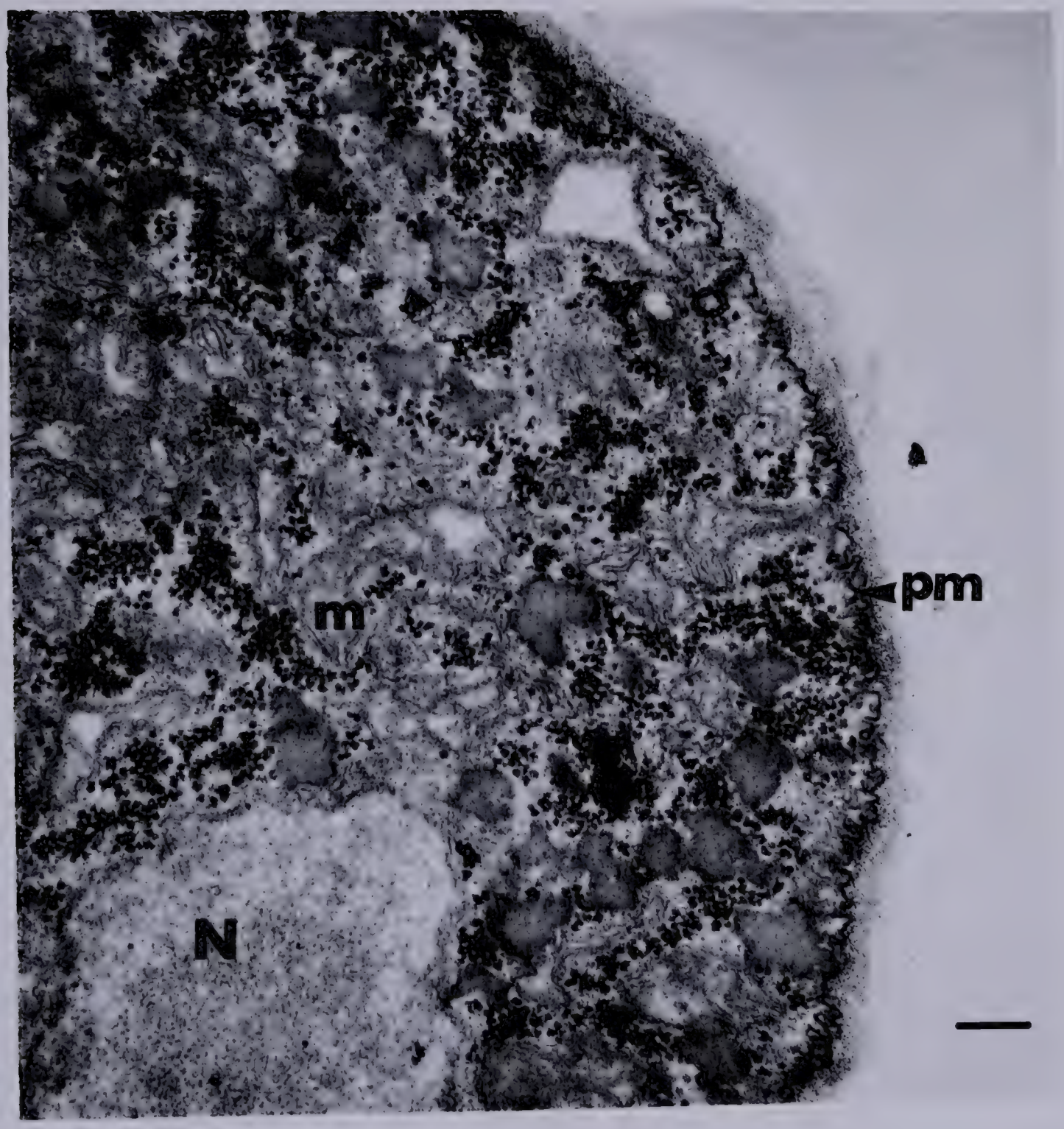

m
pm
N

FIGURE 35

A section through mouse heart ventricle fixed, after incubation in buffer at room temperature for 30 minutes, in glutaraldehyde and OsO_4. Mesosomal-like structures can be seen (arrows) in some of the mitochondria. The material is not stained, and the calibration line represents 1.0 micron.

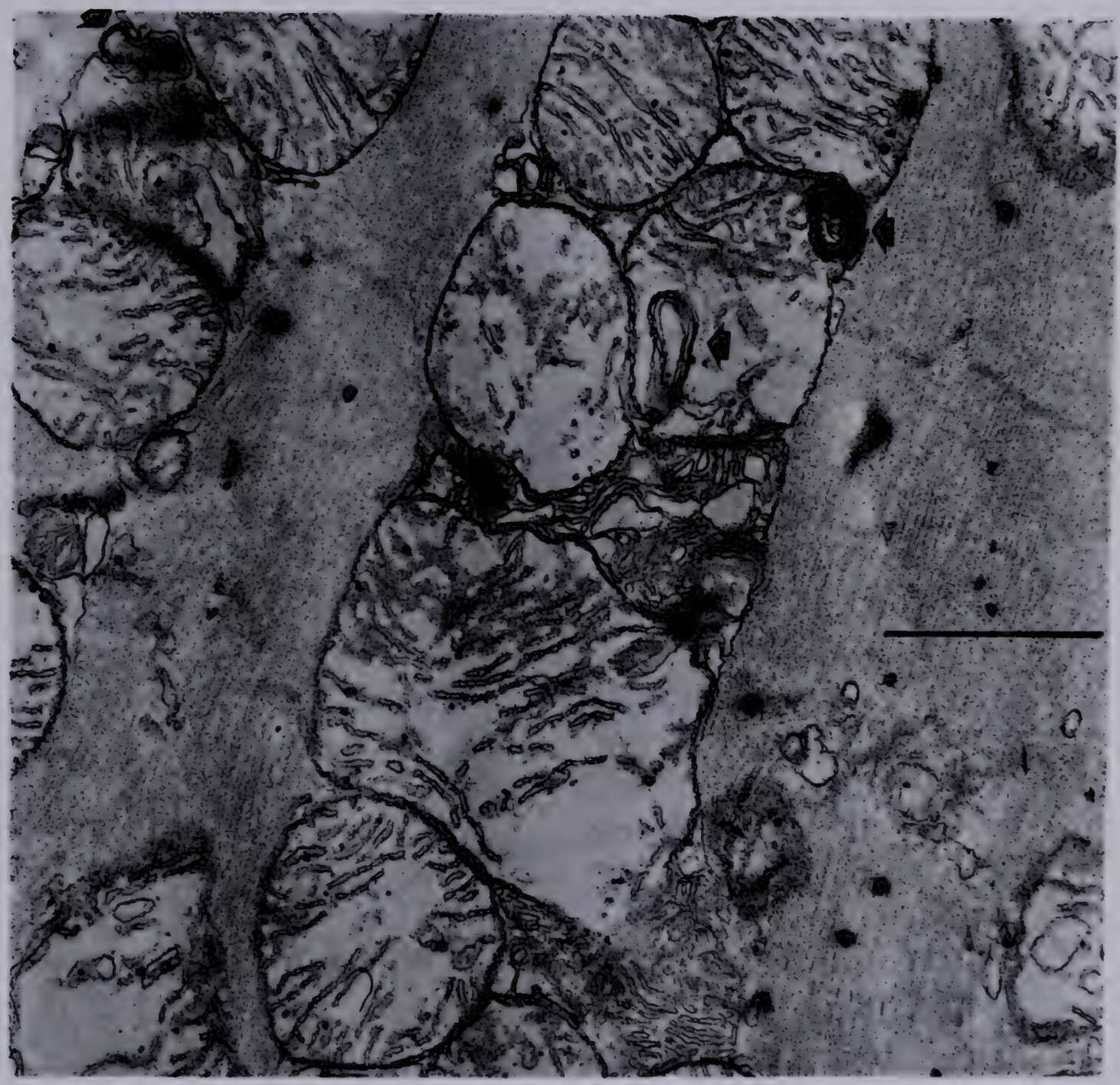


FIGURE 36

A freeze-etch prepared replica of a Neurospora cell in the exponential phase of growth. The fracture plane crossed the cell obliquely, exposing the cell wall (cw) and plasma membrane (pm) for some length. Various cellular organelles are exposed, mainly nuclei (N) and mitochondria (m). Nuclear pores may be seen on two of the nuclei. Note that the membrane surfaces appear smooth and not convoluted as seen in material fixed only in OsO_4. The calibration line represents 1.0 micron.

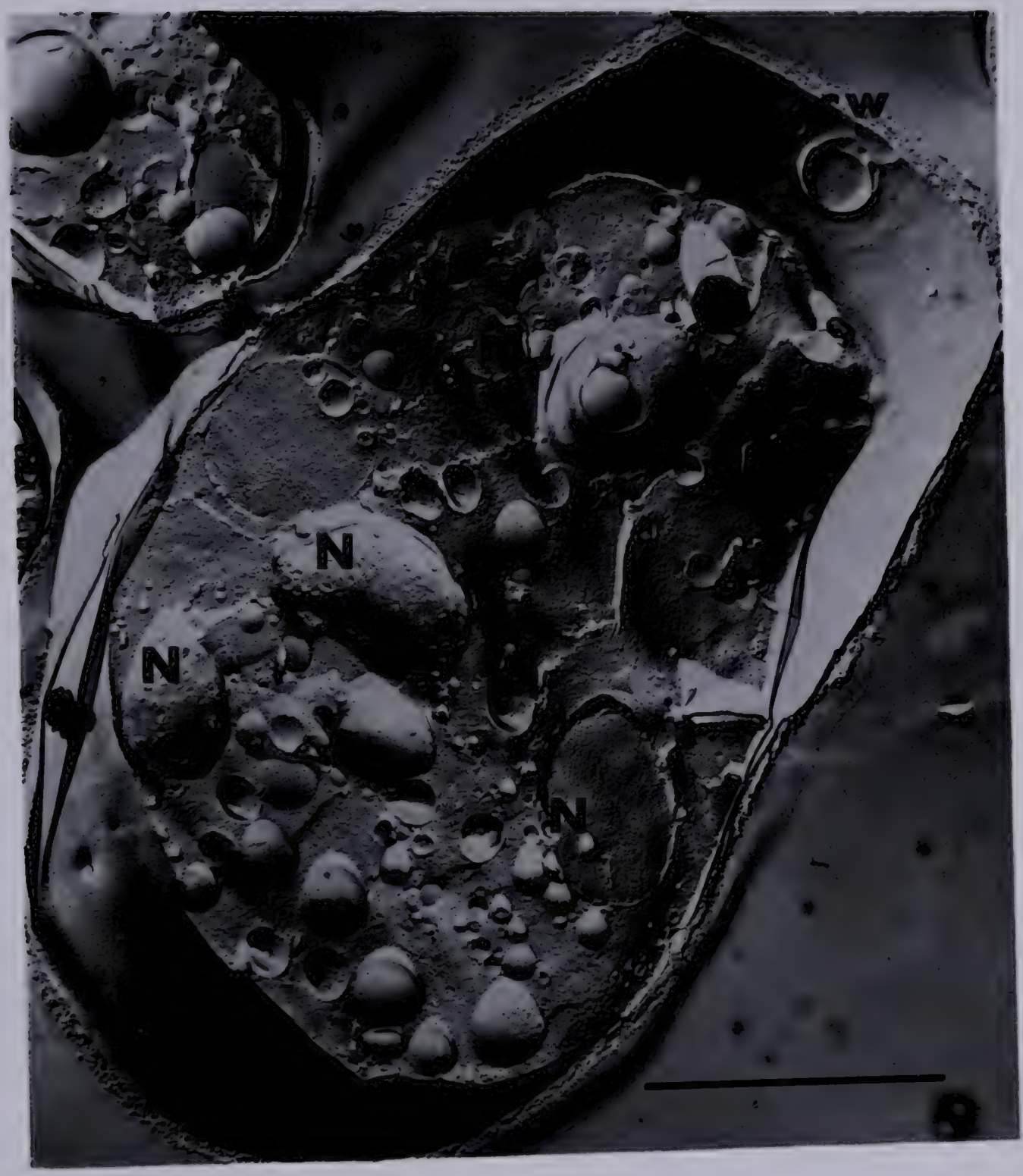
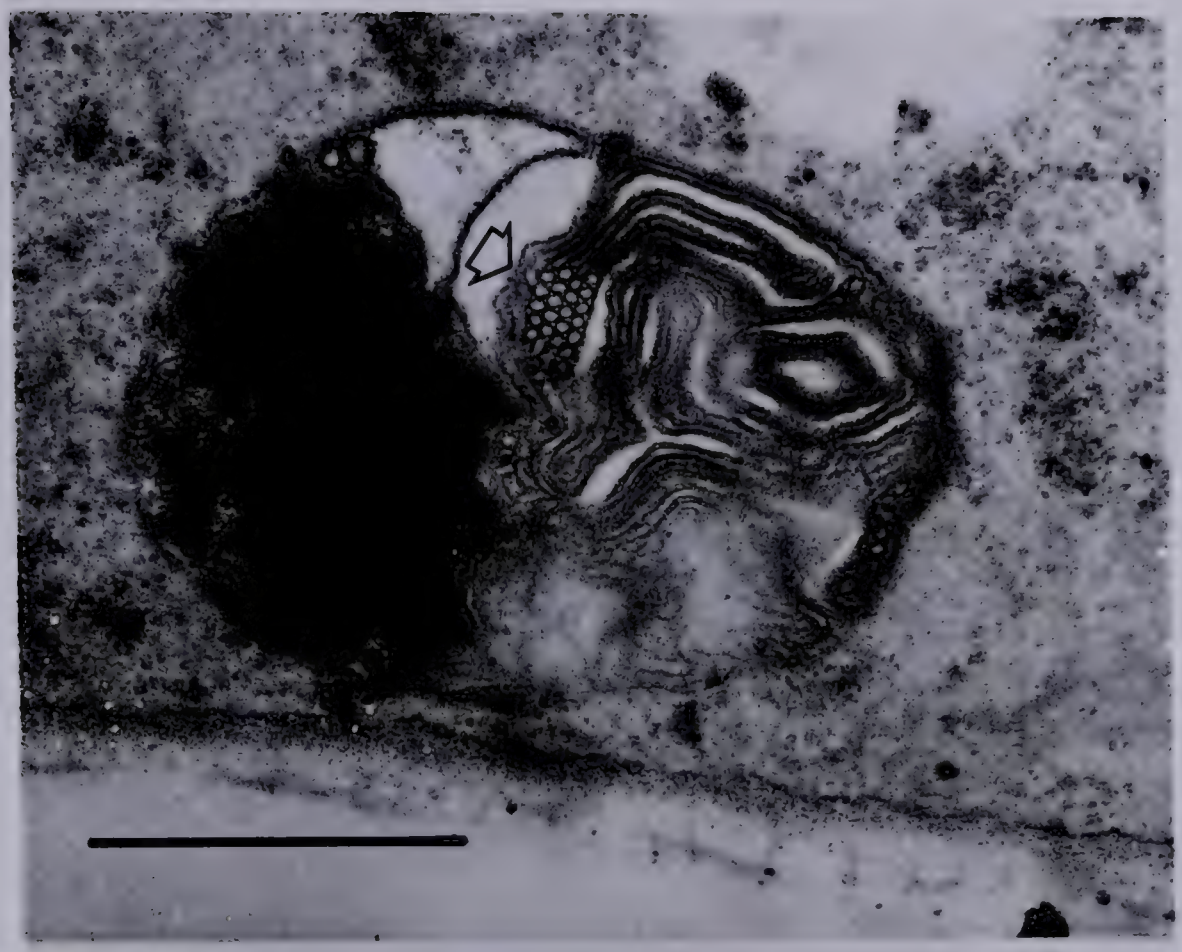

W
N
N'
N
S

FIGURE 37

Electron micrographs of a Neurospora cell doubly fixed from the exponential phase of growth. A well developed mesosome is shown in the upper micrograph. Apparent reorganization of the unit membrane in this mesosome can be seen (arrow). The lower micrograph is the same mesosome photographed at higher magnification. The small arrows indicate the unit membrane structure of the mesosomal membranes, and the large arrow indicates a region in which reorganization of the unit membrane to a tubular array has apparently occurred. The material is stained with uranyl acetate and lead citrate. The upper calibration line represents 0.5 micron, and the lower 0.1 micron.



II. Functional Studies

A. Respiratory Activity

The results of the histochemical investigations indicate the presence of an activity which oxidizes succinate and concomitantly reduces an electron acceptor (ferricyanide or TNBT). This activity is visualized in electron micrographs in association with mesosomes and the plasma membrane, as well as the mitochondrial membranes (Fig. 19). The dependence of this activity on the presence of succinate, and its inhibition by malonate, indicate that the activity is dependent upon the presence of a succinic dehydrogenase.

Assays of SDH (succinic dehydrogenase) activity in fractions from Neurospora show approximately 8 % of the total SDH activity of a given volume of cell free extract to be associated with the microsomal fraction. Electron microscopical examination of the microsomal fraction, and the distribution of F_1 ATPase activity in the fractions, indicate that cross contamination of the microsomal fraction by mitochondrial material is low. The amount of mitochondrial contamination, as measured by F_1 ATPase activity, is considerably less than the amount of SDH activity found in the microsomal fraction. Therefore, it seems that mitochondrial contamination of the microsomal material could not account for the entire SDH activity observed in the microsomal fraction.

The extra-mitochondrial finding of SDH activity is not unique to Neurospora. Two different cytoplasmic succinic dehydrogenases (or fumarate reductases) have been demonstrated in yeast (40). McEwen et al. (41) have presented evidence for a unique nuclear SDH activity in calf thymus nuclei. Also, the results of the parallel studies of SDH activity in rat liver cells reported above indicate the presence of extra-mitochondrial SDH activity in these cells, or at least some of these cells, and that the extra-mitochondrial finding of SDH might occur generally in various kinds of cells.

Riess (42) reported that the ferricyanide technique is not applicable to Neurospora because of inhibitory action of $CuSO_4$ and $K_3Fe(CN)_6$ on its SDH activity. He found 95 % inhibition of tetrazolium reduction by succinate in intact mycelia in the presence of 3 umoles/ml $CuSO_4$, the amount present in the ferricyanide medium. Reiss also stated that, even after prolonged incubation in the ferricyanide medium, distinct granular reaction products are not observed. However, the reaction product produced by incubation of Neurospora in ferricyanide medium is not discernable with the light microscope, but considerable amounts of electron opaque reaction product can be easily visualized in sections of mycelia processed for electron microscopy. Shepard (43) reported that Cu^{++} is inhibitory to SDH activity of Neurospora in cell free extracts when methylene blue is used as

the electron acceptor. This apparent contradiction from the results presented in this paper, in which reduction of ferricyanide in the presence of Cu^{++} has been observed in situ, could result from acceptance of electrons at different levels in the respiratory chain by the different acceptors. Singer and Kearney (18) indicate this possibility in their extensive discussion of SDH from various sources.

Demonstration of SDH activity associated with mesosomes and the plasma membrane suggests that these membranes could be involved in mitochondrial biogenesis. Neurospora cells lack an endoplasmic reticulum, and modifications of the plasma membrane could substitute partly for this system. One of the functions of this system may be to transport mitochondrial components from the site of synthesis to the mitochondria. Evidence for continuity between the endoplasmic reticulum and the outer mitochondrial membrane has been presented by several authors (44, 45, 46), and transport of newly synthesized proteins from the endoplasmic reticulum (microsomal fraction) to mitochondria has been shown in rat liver cells (8).

Singer and Kearney (18) indicate that acceptors such as ferricyanide, TNBT, methylene blue, and 2, 6-dichlorophenolindolphenol do not accept electrons directly from SDH, but rather from other components of the respiratory chain one or more steps removed from SDH. They also show that these

compounds accept electrons poorly, or not at all, from purified preparations of SDH. This suggests that the histochemical and biochemical assays employed to detect SDH activity in Neurospora determine not only the presence of SDH activity, but of one or more other respiratory components as well. As SDH activity has been detected associated with extra-mitochondrial membranes in Neurospora by these methods, this could suggest that SDH and other respiratory components are pre-assembled outside the mitochondria on a membrane system, and then incorporated into the mitochondrial membrane as a unit.

B. Mitochondrial DNA Replication

The results of studies on factors influencing in vitro incorporation of ^{3}H-TTP into mitochondria of Neurospora demonstrate that the chief factor affecting this activity is the presence of a nuclease activity associated with the mitochondrial fraction. This nuclease activity rapidly degrades E. coli DNA. A similar degradation of newly synthesized DNA could occur within the mitochondria. The nuclease activity appears to be specifically associated with mitochondria, and is similar in its characteristics to the nuclease described by Linn and Lehman (33) from mitochondria of Neurospora. They found that the nuclease could not be washed free of the mitochondria, that purified mitochondrial

nuclease was not bound by isolated mitochondria and, upon sonic disruption of the mitochondria, a significant increase in nuclease activity occurred. All this evidence suggests an intra-mitochondrial location of the enzyme. Moreover, it has been reported that mitochondria of Neurospora are impermeable to DNAse (47). Linn and Lehman isolated and characterized this enzyme as an endonuclease, active on both single and double stranded nucleic acid to an equal extent. The enzyme requires a divalent cation (Mg^{++}, Mn^{++}, Co^{++}, but not Ca^{++}), is inhibited by EDTA, and has a pH optimum of 6.5 to 7.5 and a temperature optimum of 37°C to 45°C. These properties distinguish this endonuclease from the extra-mitochondrial nuclease first described by Linn and Lehman (48) and by Rabin et al. (49). The decrease observed in incorporation of ^{3}H-TTP by mitochondrial fractions of Neurospora upon storage could be accounted for by the parallel increase in nuclease activity conspicuous in these fractions. However, the relationship between the DNA polymerase activity and the nuclease is not clear.

Degradation of the newly synthesized DNA by the nuclease should produce a small amount of ^{3}H-TMP. However, the amount of ^{3}H-TMP observed upon assaying was far greater than one would expect to result from the nuclease activity. Moreover, in the absence of incorporation of ^{3}H-TTP by mitochondria, even a greater amount of ^{3}H-TMP was recovered. These observations suggest that there is an enzymatic activity

responsible for the conversion of ^{3}H-TTP to ^{3}H-TMP. This activity may be due to the presence of kinases associated with the mitochondria.

The amount of ^{3}H-TTP incorporated by mitochondrial fractions of Neurospora is probably the net result of the three enzymatic reactions discussed above. Two of these reactions may compete for the same substrate, i.e., ^{3}H-TTP, and the third reaction may degrade the newly synthesized product, i.e., DNA, as it is formed. These reactions make it impossible to obtain labelled mitochondrial DNA with high specific activity by this method. Because of the lability and low activity of the mitochondrial DNA, autoradiography was not attempted. The detergent fractionation method of Tremblay et al. (10) was attempted to elucidate a possible role of membranes (mesosomes) in mitochondrial DNA replication.

The results of detergent fractionation of Neurospora mitochondria suggest that the detergent sodium lauroyl sarcosinate had an activating effect of the mitochondrial nuclease of Neurospora. This activating effect is probably related to disruption of the mitochondrial membranes, as an increase is also seen with sonic disruption or storage of these fractions (Fig. 27). The fact that the mitochondrial DNA is found in the membrane-detergent complex after fractionation and nowhere else, strongly suggests that

mitochondrial DNA in Neurospora is membrane associated. Experiments by Tremblay et al. (10) have shown that preformed membrane-detergent crystal complexes will not bind free DNA, and work by Kalf and Faust (50) has shown mitochondrial DNA polymerase activity to be associated with the inner membrane in rat liver mitochondria. It has been shown in bacteria that membrane association is necessary for replication of DNA, and that the replicating enzymes are probably membrane bound (51, 52). Thus it seems likely that mitochondrial DNA in Neurospora is associated with some membrane, probably the inner mitochondrial membrane.

Demonstration of the possibility of mitochondrial DNA replication being membrane associated in mitochondria of Neurospora unfortunately supplies no useful information about mesosomes in Neurospora. As previously indicated, the mesosomes of Neurospora are probably reorganization products induced by fixation of the plasma membrane or some other simpler membranous structure derived from the plasma membrane. These membranes may have a role in mitochondrial biogenesis, but it seems unlikely that they are directly involved in mitochondrial DNA replication.

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